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VOL. 29 AUGUST, 1951 NUMBER 4

STUDIES ON FUNGUS PARASITES OF STRAWBERRY LEAVES IN ONTARIO[1]

BY JOAN FALL

Abstract

Four fungi were isolated from lesions on strawberry foliage in Ontario during the growing seasons of 1948 and 1949. They are *Mycosphaerella fragariae* (Tul.) Lindau, *Diplocarpon earliana* (Ell. & Ev.) Wolf, *Dendrophoma obscurans* (Ell. & Ev.) H. W. And., and *Gnomonia fructicola* (Arnaud) n. comb. (*Zythia fragariae* Laibach), and they cause the diseases known as leaf spot, leaf scorch, leaf blight and leaf blotch respectively. Apparently this is the first report of *Zythiae fragariae* in North America and of *Dendrophoma obscurans* in Canada. From field surveys, strawberry varieties were found to vary in resistance, especially to spot and scorch, and marked differences in seasonal distribution of the diseases were also evident. Spot was most prevalent in the spring and fall; scorch increased in severity during the warmer months; and blight became prominent after the cropping period. As a result of inoculation with *M. fragariae* only the middle-aged leaves of vigorously growing strawberry plants developed lesions. Premier plants, although resistant to spot in the field, proved susceptible when artificially inoculated. Penetration by *M. fragariae* may occur without lesion formation if the temperature is below 15° C. Symptom expression was best within the 20°–25° C. range. Adding strips of strawberry leaves to a suspension of conidia of *Diplocarpon earliana* greatly enhanced spore germination. Only leaves which are old or middle-aged develop scorch lesions, and these may be blotchy or diffuse depending on the variety. *Dendrophoma obscurans* appears to attack mainly the older leaves near the margins. The conidiophores are long and branched, the conidia slightly pointed, and the pycnidia are dark, leathery, and clumped in culture. *Zythia fragariae* has simple conidiophores, biguttulate conidia with rounded ends, and soft light brown pycnidia. The name *Gnomonia fructicola* is proposed for the fungus *Gnomonia fragariae* Klebahn form *fructicola* Arnaud because it is considered to be a different species from *Gnomonia fragariae* Klebahn.

Introduction

In the strawberry growing areas of Ontario, confusion exists with respect to the delineation of the strawberry foliage diseases which occur and the symptomatology which characterizes them. In the hope of clarifying this situation, field surveys have been made with concomitant isolations of pathogens associated with the different disease expressions which were found and

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Contribution from the Department of Botany, University of Toronto, Toronto, Ont., and contribution No. 1073 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada. Based on a thesis presented in April, 1949, to the University of Toronto in partial fulfilment of the requirements for the degree of Master of Arts.

[The June number of this Journal (Can. J. Botany, 29 : 159-297. 1951) was issued June 29, 1951.]

subsequent infection experiments with the fungi thus isolated. From the results obtained, it has become apparent that, within this area, four foliage diseases of strawberry occur commonly. They are leaf spot, leaf scorch, leaf blight, and leaf blotch, and are caused by *Mycosphaerella fragariae* (Tul.) Lindau, *Diplocarpon earliana* (Ell. & Ev.) Wolf, *Dendrophoma obscurans* (Ell. & Ev.) H. W. And., and *Zythia fragariae* Laibach respectively. A summary of the field surveys and of the experiments related to each of these diseases is presented in this paper.

Distribution of Relative Importance

Plantations in the Ottawa and St. Catharines areas were surveyed during the growing seasons of 1948 and 1949 to study the symptom expression and to determine the distribution and relative importance of spot, scorch, and blight. Marked seasonal trends in the distribution of these diseases were encountered. Spot was prevalent in the spring and again in the fall and was much less so during midsummer. Scorch did not become prevalent until midsummer, whereas blight was most evident in late summer and the fall. However, while these distributional trends were pronounced and consistent, as early as May in both areas all three fungi were present and sporulating. The three diseases may, therefore, be encountered at any one time in the same area.

From the surveys, it was apparent that the symptoms of these three diseases varied in severity and in expression, depending on the variety of strawberry being examined. In many cases, the symptom expression of the different diseases was so very similar that diagnosis on this basis was difficult, and this was especially true in the initial stages of disease development when the lesions caused by all three fungi were found to begin as virtually indistinguishable purple spots.

In the aggregate, these three diseases are of economic importance in all the strawberry growing areas of the province. The variety Premier, which is grown predominantly in the Clarkson–Niagara area, is fairly resistant to spot and scorch, but becomes severely affected by blight after the cropping period. Valentine, a relatively new variety from the Vineland Horticultural Experiment Station, is beginning to take the place of Premier in many areas, and since it is susceptible to spot, the economic importance of this disease is likely to be enhanced. In the Ottawa area, all three fungi are prevalent on the varieties grown.

The blotch fungus was isolated on several occasions from lesioned leaves in the Ottawa district. The perfect stage, which proved to be a species of *Gnomonia*, was found on overwintered strawberry leaves in the spring. The actual distribution and relative importance of this disease has yet to be determined. It has been found frequently associated with *Dendrophoma obscurans* with which it has undoubtedly been confused. Further investigation and surveys made with this fact in mind will be necessary to clarify the picture with respect to both blight and blotch.

Leaf Spot of Strawberries

Mycosphaerella fragariae (Tul.) Lindau produces the disease commonly known as strawberry leaf spot. It is usually collected in the imperfect or *Ramularia* form, but the black perithecia have been found on lesions on overwintered leaves in early spring (Figs. 1 and 2). Diseased leaves, if kept in a moist chamber overnight, develop over each mature lesion a field of conidiophores bearing spores at their tips (Figs. 3 and 4). On most susceptible varieties, the lesions begin as purple spots, the centers of which later become necrotic and turn white. In this way a lesion is formed which is one-eighth to one-quarter of an inch in diameter and has a sharply defined light center surrounded by a purple border.

Varietal Symptom Expression

The variety Senator Dunlap, which is still grown extensively in the Ottawa region, is very susceptible to spot (Fig. 5). Typical lesions, as described above, occur generally, but often the lesions are much larger and tend to spread until a considerable leaf area is involved. The center or centers of a large lesion may remain a light brown rather than turn white. The variety Louise also produces this type of spreading lesion in the field.

Mycosphaerella fragariae produces quite a different type of lesion on *Fragaria nipponica* Mak. The spots look at first like oval drops of light brown paint over the bright green leaves. Only in the very old lesions is any dark border visible.

The variety Premier very rarely shows any spot lesions. However, when much inoculum is present, as when Premier plants are grown next to a very susceptible variety, an occasional lesion may be found on plants in the adjacent row (Fig. 6). Where these occur, they are of the same type and size as on susceptible varieties.

Typical spot lesions were found on Valentine, Fairfax, and Temple varieties growing in the Niagara region, and also on many of the varieties and seedlings of the Vineland Horticultural Experimental Station and of the Division of Horticulture, Ottawa. Striking evidence of varietal resistance was seen in the plots at these stations where resistant seedlings, growing next to badly infected seedlings, remained free from disease or were only slightly spotted.

Studies of Mycosphaerella Fragariae

(*a*) *Effect of Temperature on Growth in Culture*

Spores are produced readily in culture. The colonies spread very little, but rather build up and become stromatic and dense. Sporulation and mycelial growth occur simultaneously on potato dextrose agar, and in these studies "growth" refers to the combination of both phases of development. Plates were inoculated with a loopful of spore suspension and incubated for five days at temperatures ranging from 5° to 35°C., at which time the growth was compared photographically (Fig. 7).

From this, it was evident that there was no growth at 35° C., little more than germ tube formation at 30°, and most growth at 25° C., while below this temperature growth gradually decreased, but was still evident at 5° C.

(*b*) *Effect of Humidity on Spore Germination*

Leaves bearing fruiting lesions were washed under running water, using a clipped camel hair brush to remove all the old spores. The leaves were allowed to dry and then were placed in a moist chamber at room temperature. The following day a new crop of spores was produced on the lesions. This method is dependable and gives a supply of spores of uniform age. Distilled water was used to make the spore suspension, drops of which were placed on clean slides for germination tests.

The procedure outlined by Hopp (5) was used to create atmospheres with relative humidities varying from 90 to 100% at 1% intervals. As soon as the water had evaporated from the drop of spore suspension on the slide, the slide was placed in a small open dish which floated in its respective humidity chamber. The spores were examined after 24 hr. No germination occurred below 98% relative humidity. The spores appeared normal at 97 and 96%, but at 95% they were shrivelled. At 100% long straight germ tubes were produced; at 99% the germination was good, with slight curling of the tubes. At 98% most of the tubes were short, but the few long ones which were produced were coiled.

In the experiment just reported, drops of spore suspension were placed directly in a relative humidity of 100%. On examination later it was found that in such cases 98–100% of the spores germinated, whereas when the drops were allowed to evaporate and then were placed in 100% relative humidity, germination was only 80–82%. The reason for this difference is not apparent and it has not been possible to investigate the matter further.

(*c*) *Effect of Temperature on Spore Germination*

A crop of spores of the same age was obtained by the method already described. Petri plates were used as moist chambers and a spore suspension

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FIG. 1. Perithecia of *Mycosphaerella fragariae* scattered over a lesion on an overwintered Senator Dunlap strawberry leaf.

FIG. 2. Conidia of *M. fragariae* being produced from the germinating ascospores within the perithecia. The ascospores of this fungus are not disseminated.

FIG. 3. A new crop of conidia of *M. fragariae* being formed after a washed leaf was kept for 24 hr. in a moist chamber at room temperature. The conidia are confined to the light center of the small lesion.

FIG. 4. Conidia over the surface of a spreading type of spot lesion found on Senator Dunlap plants.

FIG. 5. Leaves from a badly spotted Senator Dunlap plant collected near Ottawa. Lesions are spreading on this variety.

FIG. 6. Spotted leaves of Premier plants which had been growing next to a very severely diseased variety. These were the only spotted Premier leaves found in the patch. The large lesions on the leaf on the left were caused by *Dendrophoma obscurans* and the difference between blight and spot lesions can be seen.

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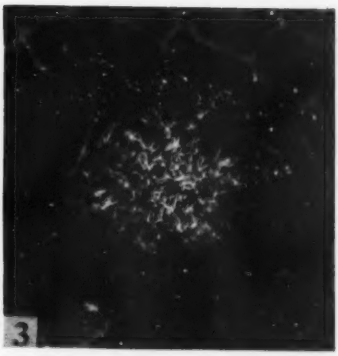
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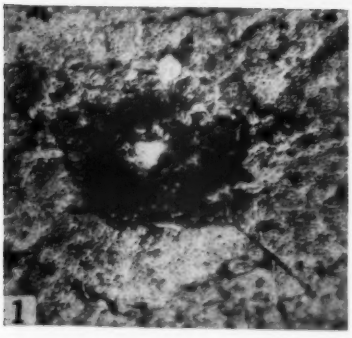
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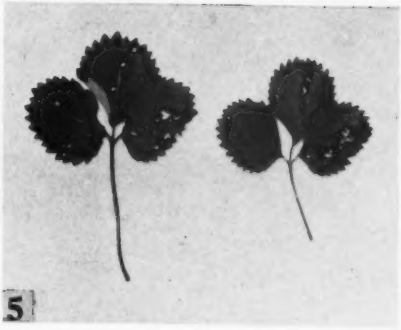
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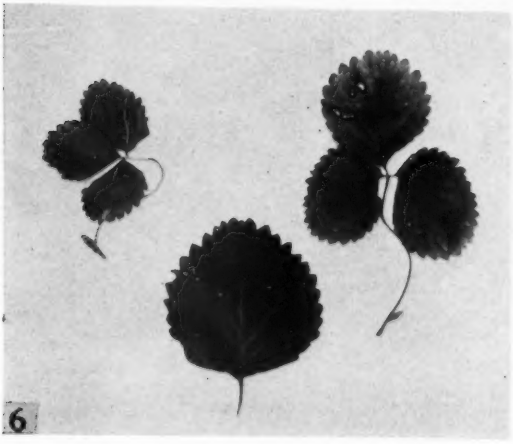
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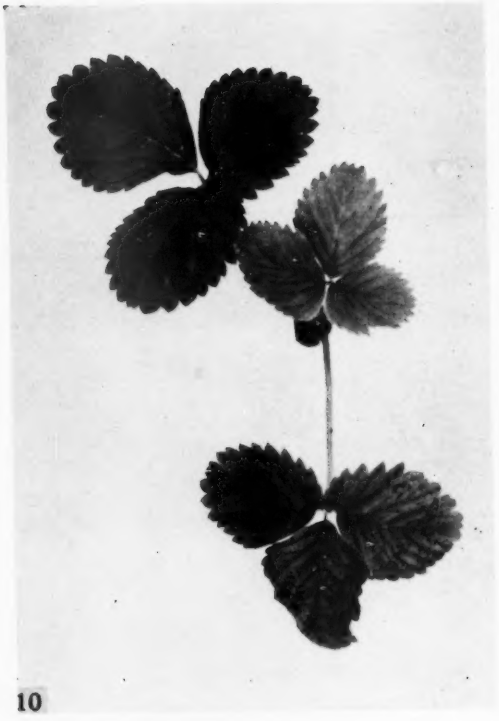

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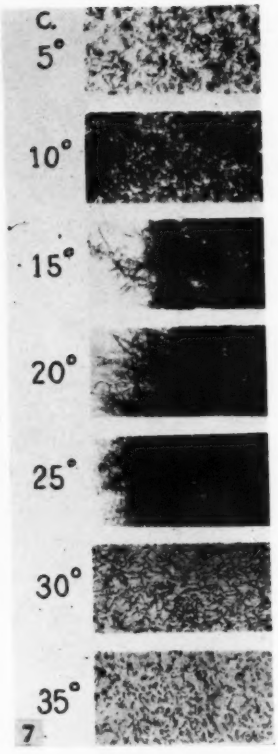

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was made with distilled water. Loopfuls of suspension were placed on clean slides which were put in the Petri plates. The plates were then placed at temperatures varying from 5° to 35° C. They were examined after 24 hr.

No germination was visible at 5° C., even after three days, but the spores appeared perfectly normal. At 35° C. there was also no germination, but the contents of the spores had become coagulated. When this slide was placed at 15° C. for another 24 hr., no germination occurred, indicating that the spores had been killed at 35° C. Buds, 8 μ in length, were formed at 10° and 30° C. The maximal germination occurred at 15° C., and at 20° C. was very nearly as good, the germ tube lengths being 80 and 70 μ respectively. At 25° C. the germ tubes were, on the average, shorter, 25 μ, but occasionally long ones were present.

(*d*) *Effect of Temperature on Spore Production*

Leaves bearing fruiting lesions were removed from plants, washed under running water to remove old spores, allowed to dry, and put in moist chambers. These were placed at temperatures varying from 5° to 35° C. at five degree intervals. After 24 hr., spore production was just beginning at 5° C., the conidiophores being evident. At 10° the conidiophores were sparse and tufted. This tufted type of distribution was changing to a more uniform distribution at 15°, and at 20° the lesion was uniformly covered with conidiophores. There was a dense mat at 25° C. The growth at 30° was very similar to that at 10°, the conidiophores again appearing tufted. No spores were produced at 35° C.

Infection Studies

Three constant temperature and humidity chambers were available for studying the effect of temperature on infection and symptom expression. They were operated at 10°, 15°, and 20° C. The varieties Valentine, Louise, and Senator Dunlap, which are subject to leaf spot in the field, and Premier, which is resistant to leaf spot in the field, were tested. Wild strawberry plants, *Fragaria vesca* L. and *F. virginiana* Duchesne were used as well. The inoculum was prepared from week old cultures of *M. fragariae* isolated from several sources. This was done in the hope of including as many races of the fungus as possible, in case it should prove highly specialized parasitically. The plants were inoculated by painting both surfaces of all the leaves with a heavy spore suspension by means of a camel hair brush, and were placed at

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FIG. 7. Photomicrographs of cultures of *M. fragariae* which were grown from conidia for five days at the temperatures indicated. Growth occurred from 5° to 30° C., with an optimum at 25°, but no growth took place at 35° C.

FIG. 8. *Fragaria vesca* plant which was inoculated with conidia of *M. fragariae*, showing the specific attack of the fungus on middle-aged leaves.

FIG. 9. *Fragaria virginiana* plant showing infection by *M. fragariae* on one leaf only, although every leaf had been thoroughly covered with a spore suspension.

FIG. 10. Valentine plant showing the specific attack of *M. fragariae* on the middle-aged leaf only. The older and younger leaves are free from infection, although they received the same treatment as the infected leaf.

once in the chambers. For the first three days, a relative humidity of 100% was maintained after which it was dropped to 90% for the remainder of the experiment.

An experimental lot of plants was also inoculated and placed in the greenhouse under moist chambers for three days. At the end of this period, some were exchanged with inoculated plants from each of the chambers, and the rest kept in the greenhouse for the duration of the experiment. In this way it was hoped to test the effect of the different temperatures on penetration as well as on the symptom expression. Each experimental lot in the chambers therefore comprised 10 Valentine plants, five of which had been exchanged for five greenhouse inoculated plants, three Louise plants, one of which had been exchanged, eight wild plants, four of which were exchanged, two Premier, two Senator Dunlap, and five check plants of each variety. The checks remained healthy throughout the experiment. In the greenhouse, likewise, there were four comparable experimental lots, one of which had been inoculated in the greenhouse and retained there, whereas the other three had been inoculated in the chambers, one at 10, one at 15, and one at 20° C. before being transferred to the greenhouse.

Symptoms began to appear within seven days at the higher temperatures, and were completely expressed within 12 days. At that time, a striking difference in disease incidence was apparent on leaves of different ages. The youngest leaves were not affected at all on any of the plants, middle-aged leaves were spotted severely, while only occasionally did spots occur on the older leaves (Fig. 8, 9, 10).

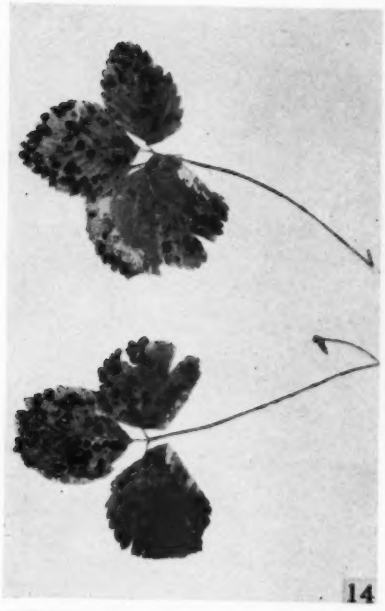
Plants removed from all three chambers to the greenhouse developed symptoms. The temperature in the greenhouse varied between 15° and 25° C. Lesions on the plants held in the 20° chamber were more spreading than those on plants kept in the greenhouse. This could have been the effect of a higher humidity in the chamber. Lesions on plants at 15° C. were slower in appearing and in developing. Two small lesions formed on plants brought from the greenhouse to the 10° chamber, but with this exception, no lesion appeared in this chamber. The temperature in this chamber was then raised to 25° C., and in five days many vigorous lesions were appearing on those plants brought from the greenhouse to 10°, while two days later, lesions also appeared on the plants which had been held at 10° from the start. Thus it is apparent that penetration took place at 10°, 15°, and 20° C., but that symptoms were expressed best between 20° and 25°, and only slightly at 15°.

FIG. 11. Young lesions caused by *M. fragariae* on a Premier plant. The fungus attacked only the middle-aged leaves.

FIG. 12. *Potentilla anserina* collected near St. Catharines, Ont., showing lesions caused by *Ramularia* sp., a fungus very similar to *M. fragariae* in culture, but which will not attack strawberry plants.

FIG. 13. Pin-point type of scorch lesion.

FIG. 14. Blotchy type of scorch lesion.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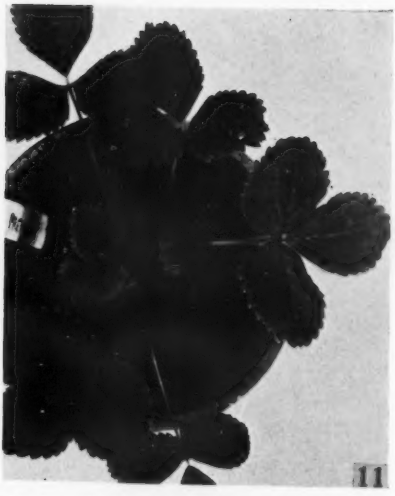
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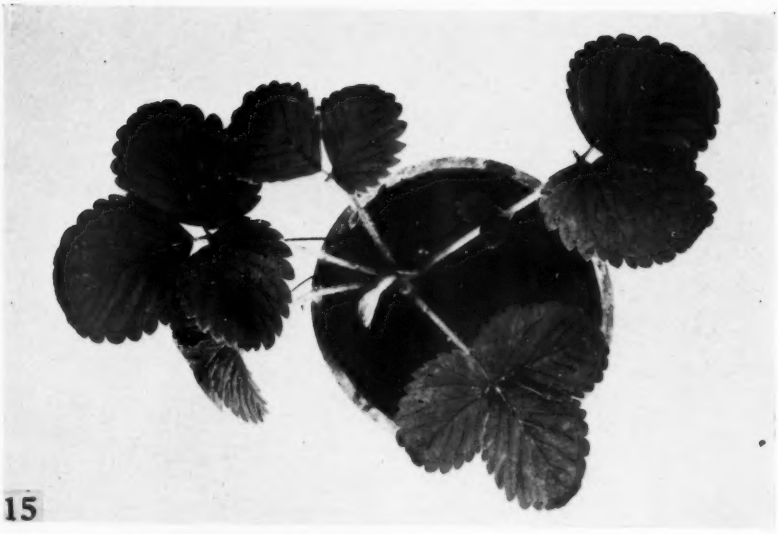

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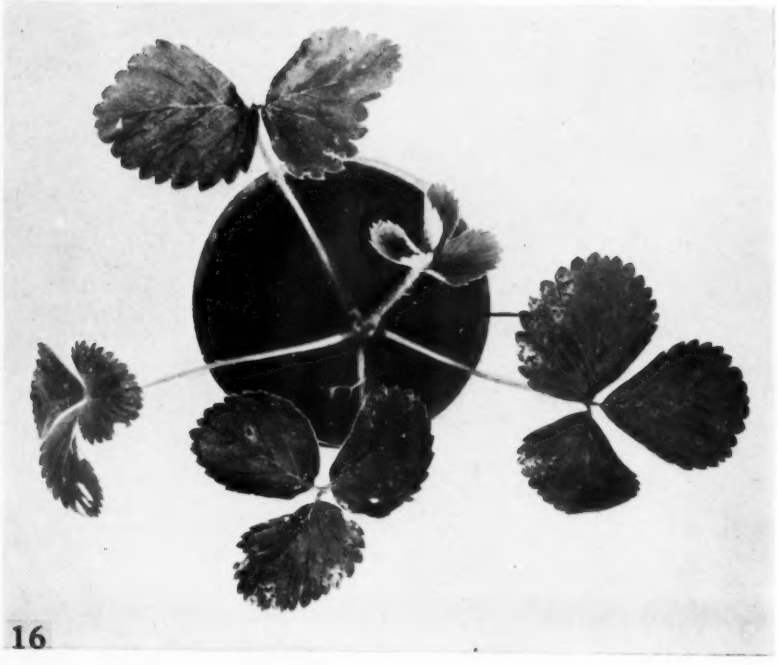

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Infection was first evidenced by small black areas on the leaves (Fig. 11). These extended and coalesced rapidly in the 20° chamber and in the greenhouse until much of the leaf was blackened. These areas began, then, to get lighter in the center. The characteristic white-centered lesions were produced in the greenhouse sooner and more consistently than at 20°, and not at all at 15° C. It would seem that the consistently maintained high humidity of the chambers delayed the whitening, and that at 15° the maturation of the lesions was delayed. Premier plants, although resistant to spot in the field, became infected under these artificial conditions as readily as did the other varieties.

Isolations of a leaf spot fungus were made from *Potentilla anserina* L. growing wild near St. Catharines (Fig. 12). These cultures seemed identical with those of *M. fragariae*, and sporulation on the host was of the *Ramularia* type. However, this fungus did not attack the strawberry varieties, but was severe in its attack on the younger leaves of *P. anserina*. *P. monspeliensis* L. is readily infected with *M. fragariae*, while *P. reptans* L. and *P. canadensis* L. were not attacked by either fungus.

Strawberry Leaf Scorch Caused by *Diplocarpon earliana* (Ell. & Ev.) Wolf

Symptom Expression

In the field, scorch lesions were found to take two forms. There were pinpoint lesions (Fig. 13), occurring in large or small numbers on the leaves, and there was also a blotchy type of lesion measuring a quarter to half an inch in diameter (Fig. 14). When severe, the lesions cause the leaves to present a scorched appearance due to the dying and drying of the infected tissue. Under suitable conditions, spores are produced in black acervuli which stand out on the upper surface of the leaves (Fig. 17). Scorch is also often severe on the petioles of leaves where girdling lesions may cause the death of the entire leaf.

On Premier, scorch sometimes produces a small lesion with a tiny white center, easily mistaken for spot. The blotchy type of lesion occurs on Valentine, and was noted at the Division of Horticulture, Ottawa, and the Vineland Horticultural Experimental Station on many seedling varieties, among which a marked variance in susceptibility was also noted. It is often very difficult to tell the blotchy type of scorch lesion from those produced by leaf blight. As these lesions become older, they dry out and turn tan color in the center, appearing very much like some of those produced by *D. obscurans*. The lesions caused by scorch, however, are usually more irregular than those caused by blight.

FIG. 15. Plant inoculated with *Diplocarpon earliana* showing the first symptoms of the scorch disease consisting of small purple spots surrounded by a chlorotic band. Middle to old aged leaves are attacked, the youngest leaves escaping infection.

FIG. 16. Scorch lesions developing on the variety Premier. The purple area extends in fine threads through the chlorotic zone.

Cultural Studies with D. earliana

Great difficulty was experienced in the isolation of this fungus. It is slow-growing, and therefore isolates are apt to be overrun with contaminants. The spores did not germinate in water consistently in any of the trials. Isolations were attempted during the summer of 1948 on a number of media, such as corn meal, potato dextrose, lima bean, and malt agars. Then in the late summer, pieces of yellow wax beans were sterilized in tubes and used as an isolating medium. One culture only was obtained this way. Later on, in November, much less difficulty was experienced in isolating and culturing the organism on P.D.A. The fungus grew well on this medium and produced acervuli and abundant conidia. The reason for the difficulty experienced in the midseason isolations is not apparent.

Spore Germination Studies

More uniform results were desired with regard to spore germination, and therefore the possibility that the leaves themselves exert an influence on spore germination was investigated. Two lots of a spore suspension were prepared from spores produced in cultures. Strips approximately 1/2 cm. wide of fresh green strawberry leaves, representing a total leaf area of about 16 sq. cm., had been placed in about 4 cc. of distilled water for about 30 min., and then removed before the suspension was made. In a second series, the suspension was made in distilled water. One of each series, set up in Van Teighem cells, was placed at temperatures varying from 5° to 35° C. at five degree intervals.

The results after 48 hr. were striking. At 5° and 10° C. in both series no germ tube was formed. However, swellings which could have been the beginnings of tubes, were present on some spores. In the distilled water series, short germ tubes were present at 15° C., while at 20° and 25° C. they were three times as long, but were shorter again at 30° C. Some spores germinated at 35° C. In the series which had contained fresh leaf pieces, a marked difference was seen over the temperature range of 15° to 30° C. The germ tubes were 5 to 10 times the length of those in the corresponding drops of the other series. Very long tubes were formed from 20 °to 30° C., the optimum seeming to be between 25° and 30° C. Some of the germ tubes were even beginning to branch. This indicates that some substance from the leaves greatly enhanced the germination of the conidia of *D. earliana*. Preliminary experiments indicated that spores were stimulated in a comparable way when germinating on the surface of uninjured leaves. No opportunity was found to pursue this matter further.

FIG. 17. Acervuli of *Diplocarpon earliana* produced on the upper surface of an infected leaf.
FIG. 18. Louise plant infected with the scorch organism. Numerous small round lesions were formed.
FIG. 19. Leaves from Valentine plants inoculated with *D. earliana* and kept for a month at the temperatures indicated. The lesions were more spreading at higher temperatures.

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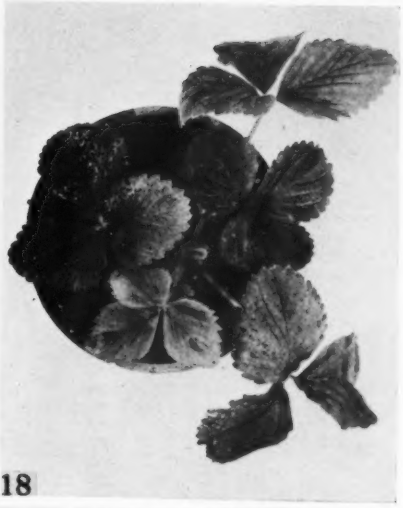

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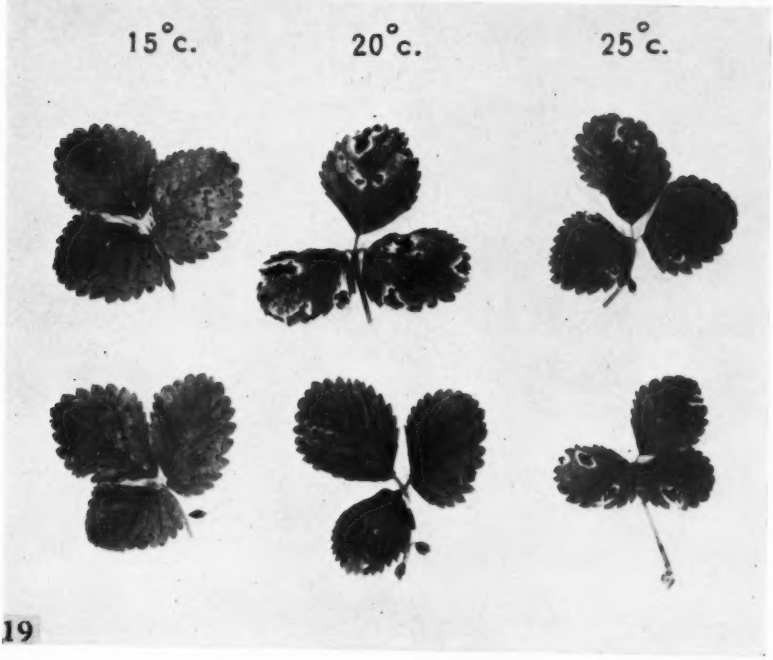

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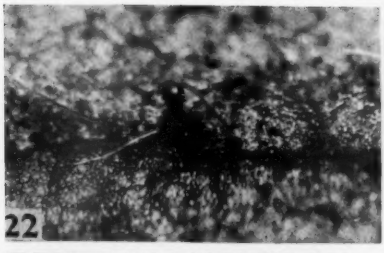
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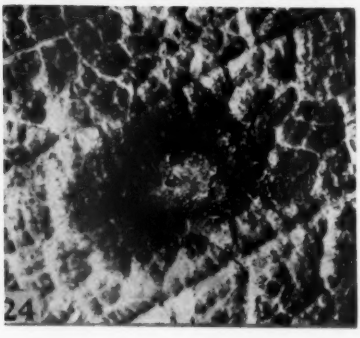
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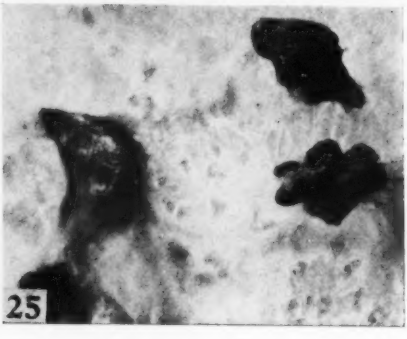
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Infection Studies

In order to test the effect of temperature on infection and symptom expression, experimental lots of plants were placed in the three chambers which were operating at 15°, 20°, and 25° C. Each lot comprised five Valentine, two Premier, two Louise, two Senator Dunlap, two *F. virginiana*, and two *F. vesca* plants. Both surfaces of all the leaves were atomized with a spore suspension made from fresh cultures and distilled water, and the plants were placed directly in the chambers. The humidity was kept at 100% for the first 72 hr., after which it dropped to around 90%.

In from seven to nine days, in all three chambers, symptoms appeared which consisted first of small chlorotic areas; these later developed a small purple center (Fig. 15). As the purple area enlarged within the chlorotic zone, the latter expanded. This process resulted in the formation of a lesion in which there was a purple area surrounded by a chlorotic band. As was the case with *M. fragariae*, the fungus did not attack the youngest leaves, but in contrast with that fungus, *D. earliana* attacked the oldest leaves as well as the middle-aged ones. The lesions spread more rapidly and fruiting occurred several days earlier on the old than on the middle-aged leaves.

Two types of lesions found in the field have already been described. Similarly, in these infection studies, two types of lesions were formed, depending on the variety affected. The spreading type of lesion developed on the Valentine variety and also on *F. virginiana*. On Premier, a diffuse type of lesion was formed, the dark area extending in threads from the central point of infection (Fig. 16). This type of lesion was formed on Senator Dunlap plants. On Louise, the lesions were similar, but more rounded (Fig. 18). On *F. vesca*, the lesions never developed past the first stage—a small black center surrounded by a chlorotic band. The symptoms were most severe between 20° and 25° C. (Fig. 19). The lesions were larger and the chlorotic band more extensive at the higher temperatures. It may be recalled here that scorch becomes most prevalent in the field during midsummer.

Strawberry Leaf Blight Caused by *Dendrophoma obscurans* (Ell. & Ev.) H. W. And.

During the summer of 1948, lesions which at first were thought to be caused by *Mycosphaerella fragariae*, were noted on strawberry leaves. Upon closer

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FIG. 20. Leaf from a Premier plant showing blight lesions caused by *Dendrophoma obscurans*.
FIG. 21. Leaves of a Senator Dunlap plant showing blight lesions.
FIG. 22. Pycnidium of *D. obscurans* beginning to swell in a humid atmosphere.
FIG. 23. Pycnidium of *D. obscurans* exuding spores in a horn under humid conditions.
FIG. 24. Lesion caused by *D. obscurans* on a strawberry leaf showing black pycnidia on the light center of the lesion.
FIG. 25. Clumps of dark pycnidia formed in a culture of *D. obscurans*.

examination, however, it was apparent that the causal organism was *Dendrophoma obscurans* (2). This was the first report of leaf blight in Canada as far as the writer can determine.

Symptoms in the Field

Young lesions caused by this fungus are generally reddish purple and almost circular. The central portions of these lesions may soon dry out, turning a brown color, or the lesion may spread considerably before this phase occurs. Discoloration occurs most rapidly along the veins. Therefore, when infection occurs here, an elliptical lesion is formed with the vein as main axis. The veins spread out in a V-shape from the central one and the discoloration often follows two veins and subsequently may involve the tissue between. In this way the V-shaped lesions, so commonly encountered in the field, are formed (Figs. 20 and 21).

Old leaves more often exhibit the disease, but younger, vigorously growing leaves, especially of the Senator Dunlap variety, very frequently are found bearing lesions. Some patches of this variety in the Ottawa area have been severely attacked.

Blight lesions are frequently strikingly similar to those of leaf spot. Each has a light area surrounded by a purple border, and, as the center dries out, the upper surface of the lesion may peel slightly and turn quite white, thus increasing the similarity to a leaf spot lesion. On some varieties also, the lesions are tardy in forming lighter centers and may be quite spreading. This type is difficult to distinguish from the spreading type of scorch lesions. Thus, in the field, blight may resemble spot on one hand and scorch on the other.

Description of the Pathogen

D. obscurans has been found sporulating in the pycnidial stage during the entire summer, but pycnidia are more often collected on the lesions in late summer and fall. They appear as small black dots, barely visible to the naked eye, and sunken in the tissue of the leaf (Fig. 24). Under humid conditions, the pycnidia swell and exude spores in a horn (Figs. 22, 23). The spores are oblong 7 $\mu \times 2\ \mu$, with a tendency to have pointed ends; vacuoles are often present at either end or near the middle of the spore. The conidiophores are much branched (Text-fig. 5). Up to this date no perfect stage has been found in the field or produced in culture. Apparently the fungus overwinters in the pycnidial stage or in the leaf tissue.

Corn meal, oatmeal, lima bean, potato dextrose, malt, and prune agars have been used to culture the organism. Pycnidia are produced in clumps on all these media (Fig. 25). They are dark, beaked, and have leathery walls. In culture, the spores are exuded in a yellowish mass. There is very little aerial mycelium produced.

Infection Studies

Experimental lots of plants were placed in each of the three chambers set at temperatures 15°, 20°, and 25° C. Valentine, Louise, Senator Dunlap,

Premier, *F. vesca*, and *F. virginiana* were used. Both surfaces of every leaf were painted with a spore suspension. Within a week, lesions were appearing in all three chambers, but infection was not at all consistant.

F. vesca seemed particularly susceptible. Most of the infections occurred near the edges of the leaves and produced the V-shaped type of lesion. The disease appeared to be more severe at 25° C. in that the lesions were more spreading. At 20° and 25° C., plants of the Valentine variety produced lesions on the middle-aged leaves very similar to the field type of leaf spot lesion, but pycnidia later appeared in the lesions. The reason for the inconsistent infection and for the appearance of these spotlike lesions is not apparent and further experiments are required on this phase of the problem.

Strawberry Leaf Blotch Caused by *Gnomonia fructicola* (Arnaud) n. comb.

In the summer of 1948, a pycnidial fungus with a perfect stage, identified as a *Gnomonia* sp., was isolated on several occasions from lesioned strawberry leaves which were at first thought to be attacked by *Dendrophoma obscurans*. It was not apparent whether the *Gnomonia* sp. was causing the trouble or was following some other fungus, such as *D. obscurans*, through the tissue.

In the spring of 1949, perithecia of a *Gnomonia* type were discovered on overwintered strawberry leaves. Isolations made from these bodies yielded cultures identical with those of the fungus previously isolated from the leaf lesions. Cultures from pycnidiospores developed perithecia readily while cultures from the ascospores produced pycnidia. The imperfect stage was identified as *Zythia fragariae* Laiback. As far as the writer is aware, this fungus had not been reported previously for North America. The perfect stage was identified as *Gnomonia fragariae* Klebahn, form *fructicola* Arnaud [Arnaud & Arnaud (3)]. Except for the paper by Alexopoulos and Cation (1) in which they apparently describe this fungus from strawberry fruit as the perfect stage of *D. obscurans*, no further reference to *Gnomonia* sp. on strawberry has been found in the North American literature. No routine examination of the berries has been made in an attempt to locate the fungus on this part of the host.

History of the Disease

In 1941, Wormald and Montgomery (11) reported a disease on strawberry foliage, the causal organism of which they identified as *Phyllosticta grandimaculans* Dubak & Krieger [Dubak (4)]. In 1944, however, Wormald (10) reidentified the fungus as *Zythia fragariae* Laibach (7) for reasons stated in his paper. Wormald regarded the perfect stage of *Zythia fragariae* as a *Gnomonia* sp., but did not positively identify or name this stage. The symptoms which he described for the disease are very similar to those of leaf blight caused by *Dendrophoma obscurans*. *Z. fragariae* has been found in England on both leaves and fruit (8) of the strawberry.

In 1918, Klebahn (6) described a fungus on strawberry foliage which he named *Gnomonia fragariae*. He considered this fungus to be either saprophytic or a very weak parasite on the leaves and petioles of the plants. He cultured the fungus on Salep agar, a farinaceous meal agar obtained from the dry roots of various orchids, and both in culture and on the plant he found only a *Gnomonia* stage, but no pycnidia or conidia. He described the asci as having thick walls, and the ascospores as having a filiform appendage at each end of the spore, and a conspicuous septum with a slight constriction in the wall at this point. He made no mention of the deliquescence of the ascus walls. Each perithecium had only one neck.

In 1931, G. and M. Arnaud (3) described a fungus which they isolated from strawberry fruit. They named it *Gnomonia fragariae* Klebahn form *fructicola* Arnaud. As well as being different in several respects from Klebahn's *Gnomonia fragariae*, this fungus had a pycnidial stage which was tentatively identified as a *Gloeosporium* sp. The pycnidial stage is in all respects identical with *Zythia fragariae* Laibach. The ascospores described by Arnaud do not have a conspicuous septum, have prominent globules in the spores, have no filiform appendages, and the ascus wall deliquesces.

In September, 1948, Alexopoulos and Cation (1) published a paper describing a fungus which they also isolated from strawberry fruit. They considered it to be a *Gnomonia* sp. and the perfect stage of *Dendrophoma obscurans* H. W. And. From the figures shown and the description given, it appears that their fungus is the perfect stage of *Zythia fragariae* Laibach rather than of *D. obscurans*.

Because differences are evident between Arnauds' and Klebahn's fungi, the writer is of the opinion that these should be separate species. Therefore it is proposed to raise Arnauds' form name to specific rank and call the fungus *Gnomonia fructicola* (Arnaud) n. comb. The imperfect stage of this fungus is *Zythia fragariae* Laibach.

Description of the Fungus

The bases of the perithecia are completely buried in the host tissue, the necks appearing like black hairs on the leaf surface. In culture, one to four necks may form per base and the perithecia may be scattered anywhere on or through the agar. They are carbonaceous, with globose bases measuring 450 to 600 μ in diameter, and with necks 500 to 1000 μ long (Fig. 26). The ascospores ooze from the tip of the neck in a refractive droplet (Fig. 27) as a result of the deliquescence of the asci. The asci are about 33 μ long and contain eight ascospores. At one end of the ascus, there appear two small refractive bodies.

FIG. 26. Perithecia of *Gnomonia fructicola*. In culture, more than one neck often form from one base.

FIG. 27. Perithecia of *G. fructicola*. The asci deliquesce and the ascospores escape in a refractive droplet at the tip of the beak.

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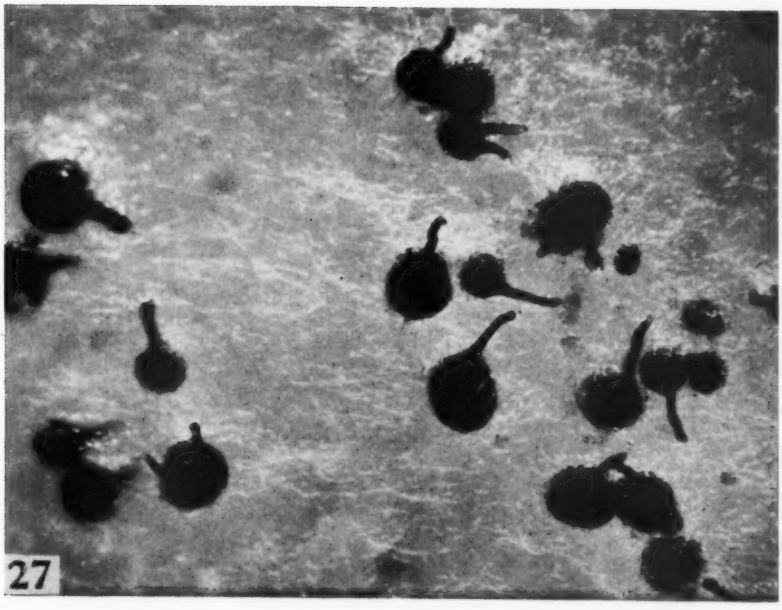

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The ascospores are at first slightly curved and contain five or six vacuoles. No septum can be seen at this stage. Apparently Arnaud saw only immature ascospores. As these mature, however, the vacuoles tend to disappear, and the spores become unequally divided into two cells with a slight constriction at the septum. They measure about 10–12 μ long and 2–3 μ wide (Text-figs. 1, 2, 3).

In culture, pycnidia of this fungus are yellowish brown in color, have a soft wall, and no conspicuous neck. The conidiophores are simple and are arranged in a palisade layer on the pycnidial wall (Text-fig. 6). The spores are 5–6 μ long and 2 μ wide. They have rounded ends and are usually biguttulate (Text-fig. 4).

This fungus, then, is easily distinguished from *D. obscurans* which has leathery dark pycnidia with a prominent neck, very pronounced branching of the long conidiophores, and spores with somewhat pointed ends. These spores are also usually biguttulate (Text-figs. 5, 6).

Cultural Studies

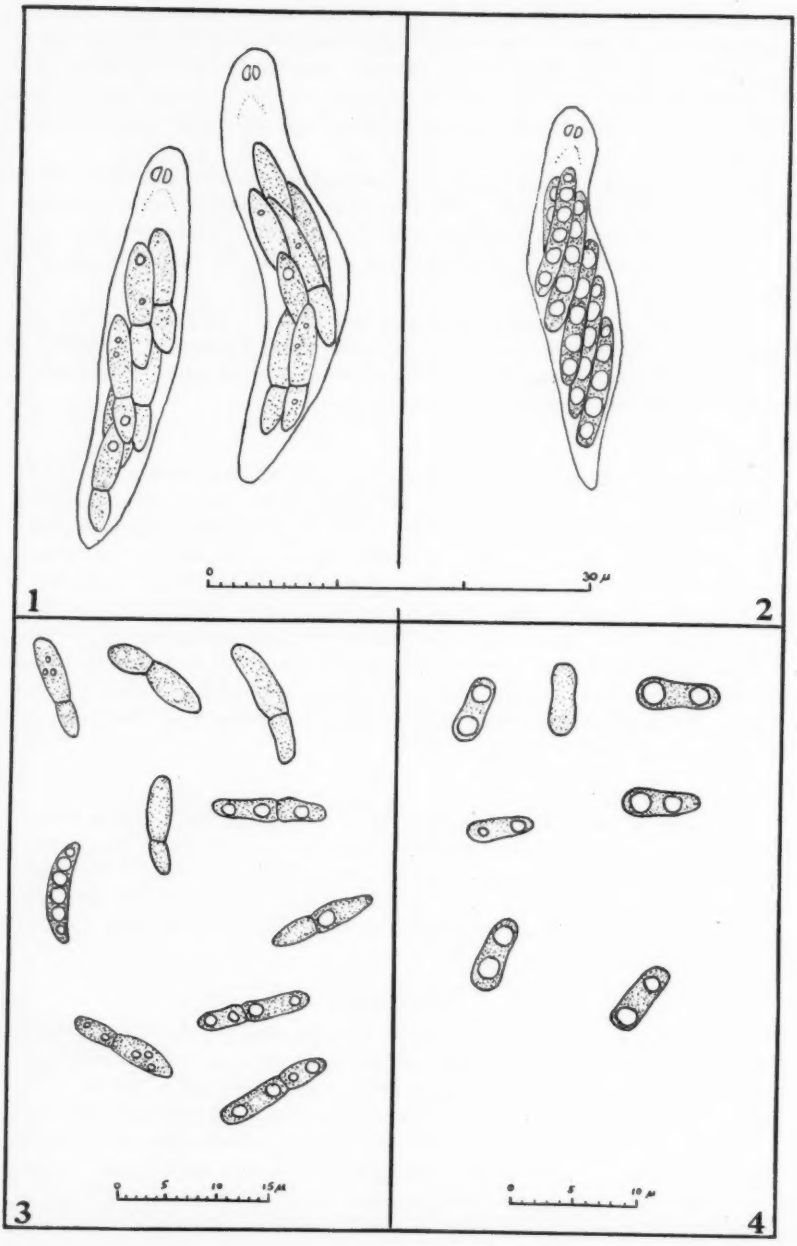
The fungus was found to grow well on P.D.A., producing abundant pycnidia and perithecia. Arnaud reported that the fungus produced abundant pycnidia on starchy media and abundant perithecia on sugary media. Many pycnidia were produced on the lima bean medium, but no perithecium was formed. On oatmeal, the production of pycnidia was abundant, but only a few perithecia were formed. On prune agar, abundant perithecia were produced, but only a few pycnidia. These experiments corroborate Arnauds' observations. It may be noted here that Klebahn grew his fungus on a starchy medium and found only perithecia, which is another indication that he was working with a different fungus. No attempts have been made to study the sexuality of the fungus or the nuclear condition of the spores.

Infection Experiments

Preliminary trials to determine the degree of pathogenicity of this fungus were not very successful. It certainly does not appear to be highly parasitic on the leaves. It can be recovered from leaf lesions, but whether or not it was the original cause of these is not too clear. During some inoculations, tiny pinpoint lesions appeared on many leaves, but even after a month these had not developed past this stage.

Discussion

Infection studies with Mycosphaerella fragariae on the varieties Louise, Senator Dunlap, Valentine, Premier, and the species *F. vesca* and *F. virginiana*, indicated that only the middle-aged leaves were infected. The size and severity of the lesions depended on the age of the leaf. All varieties used were equally susceptible under artificial conditions, although Premier is strikingly resistant in the field. The writer's results are at variance with those reported by Plakidas (9). He found that Howard 17 (Premier) had a resistance approaching immunity both in the field and when inoculated with every one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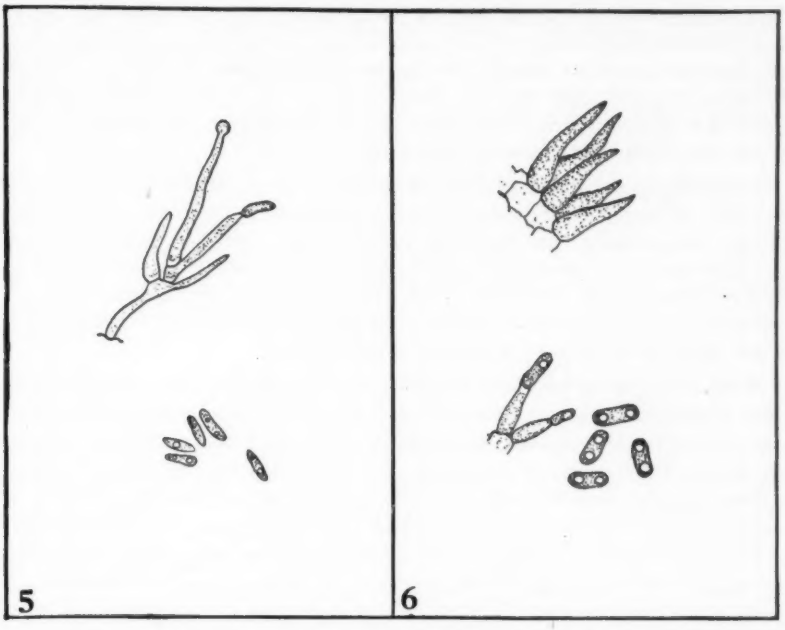
of the strains used in his experiments. Since in the experiments reported in this paper, the infection was so strikingly correlated with the age of the leaves which became infected, it seems quite possible that the apparently conflicting



TEXT-FIG. 5. Branched conidiophore and spores of Dendrophoma obscurans.
TEXT-FIG. 6. Simple conidiophores and spores of *Zythia fragariae*.

results with Premier may be explained on this basis. Unless leaves in a susceptible condition were present, no infection would occur. It seems quite possible that plants could be selected bearing no susceptible leaves at the time of inoculation. The rate of maturation of the leaves and a low inoculum potential at times critical for infection, may account partly for the striking resistance to leaf spot exhibited by Premier in the field. The experimental results seem to show that artificial inoculation might not indicate the reaction of the plant under field conditions. The desirability of further studies along this line is indicated.

TEXT-FIG. 1. Asci of *Gnomonia fructicola* showing maturing spores in which the septa can easily be seen dividing the spores unequally. Each ascus contains 8 ascospores.
TEXT-FIG. 2. Immature ascus showing immature spores. No septum can be seen at this stage.
TEXT-FIG. 3. Ascospores of *Gnomonia fructicola*.
TEXT-FIG. 4. Pycnidiospores of *Gnomonia fructicola* (*Zythia fragariae*).

Diplocarpon earliana was difficult to isolate in the summer months. The reason for this difficulty is hard to explain inasmuch as the organism seems to spread in the field during this period. The presence of strips of fresh strawberry leaves of a susceptible variety for only a half hour in a spore suspension greatly enhanced the germination of the conidiospores. Whether any strawberry leaf would do this, or whether one variety might have a greater effect than another, was not tested. Since there are roughly two types of lesions formed, pinpoint, and blotchy, there is a possiblity that those varieties forming a blotchy lesion might affect the germination of the spores in a different way from those forming the pin-point lesions.

Zythia fragariae Laibach, the imperfect stage of *Gnomonia fructicola* (Arnaud) n. comb., is easily distinguished from *D. obscurans* H. W. And. on the basis of the conidiospores, conidiophores, and pycnidia. Infection experiments did not yield conclusive evidence of the degree of pathogenicity of either of these fungi. However, *G. fructicola* seems less parasitic on the leaves than *D. obscurans*, but may prove to be highly parasitic on the strawberry fruit, since it has been found there by several different workers.

Many challenging problems for further investigation have arisen during these studies, such as: the reason for the specific attack on middle-aged strawberry leaves by *M. fragariae*, the problem of apparent field resistance to spot, the reason for strawberry leaves enhancing the germination of *Diplocarpon earliana* conidia, the symptom expression and occurrence of blight and blotch, sexuality studies of *G. fructicola*, and host–parasite relations of all four fungi. It is hoped that more research will be done on the foliage diseases of the strawberry, a crop so important in Canada to both commercial and private growers.

Acknowledgments

The author wishes to express her sincere gratitude to Prof. D. L. Bailey for suggestions, guidance, and advice during the course of these investigations; to Mr. H. N. Racicot who saw the need and encouraged the pursuit of these studies; to Dr. G. H. Berkeley for placing at her disposal the facilities of the Dominion Laboratory of Plant Pathology, St. Catharines; and to Dr. Roy Cain (University of Toronto), Mr. I. L. Conners, and Dr. J. W. Groves (Division of Botany and Plant Pathology, Ottawa) for suggestions regarding the taxonomy of the fungi.

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ACTIVITY OF ANTIBIOTICS AGAINST *ASCOCHYTA PISI*[1]

By V. R. Wallen and A. J. Skolko[2]

Abstract

A number of antibiotic substances which have been reported to possess antifungal properties were tested at various dilutions for their fungistatic activity upon spore germination of *Ascochyta pisi*. Actidione, antibiotic XG, and viridin (pH 3.5) were found to be fungistatic at dilutions of 10 p.p.m., gliotoxin and gladiolic acid at 20 p.p.m. The remaining antibiotics were less active, citronin showing no fungistatic activity even at 100 p.p.m. In fungicidal tests on spores of *A. pisi*, antibiotic XG, gliotoxin, actidione, humulon, gladiolic acid, and viridin were active at concentrations of 50 p.p.m. with a 12 hr. exposure, whereas clavacin, lupulon, and citronin required concentrations of 100 p.p.m. The action of these antibiotics upon germination of pea seed on filter paper and upon emergence in soil were tested and, in general, those which possessed the greatest fungistatic and fungicidal activity to *A. pisi* spores were the most phytotoxic. Only antibiotic XG and actidione gave adequate control of *A. pisi* when naturally infected pea seed was treated by soaking the seed in the antibiotic. Actidione, however, seriously reduced germination.

Introduction

Reports of the successful control of a number of seed-borne bacterial diseases by means of antibiotic substances (1, 2, 6, 9) suggested the possibility of a similar application in the control of seed-borne fungal diseases. To this end, several antibiotics, which have been reported as possessing antifungal properties, were screened for their fungistatic and fungicidal activity against *Ascochyta pisi* Lib. and for their suitability as a pea seed treatment. The results of these tests are presented below. On the basis of these preliminary trials, two antibiotics, actidione and antibiotic XG, were selected for further study. The effectiveness of actidione and antibiotic XG in the control of *Ascochyta* leaf and pod spot has been reported in earlier papers (18, 19). The results with these two antibiotics are included here for purposes of comparison.

Of the numerous antibiotic substances for which antifungal properties have been claimed, only a few were available for study. They are listed in Table I.

Experimental

I. Fungistatic Activity

In order to determine the dilutions at which the various antibiotics inhibited the growth of *A. pisi*, preliminary tests by means of the agar-streak method of Waksman and Reilly (17) were made. Pea agar plates at pH 5.2 containing antibiotic dilutions of from 0 to 100 parts per million were streaked with a spore suspension of *A. pisi*. Each plating was made in duplicate. The results are summarized in Table II.

[1] *Manuscript received April 25, 1951.*

Contribution No. 1102, from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

[2] *Junior Plant Pathologist and Plant Pathologist, respectively, Central Laboratory, Ottawa.*

TABLE I

LIST OF ANTIBIOTICS TESTED

Antibiotic	Produced from	Report of antifungal properties	Where obtained
Actidione	*Streptomyces griseus*	Whiffen (21) Felber & Hamner (8) Vaughn *et al.* (16) de Zeeuw & Vaughn (7) Gottlieb *et al.* (10)	Upjohn Co., Kalamazoo, Mich.
Antibiotic XG	*Bacillus subtilis*	Lewis, Hopper, & Shultz (13) Hobby *et al.* (12)	Chas. Pfizer Co., Brooklyn, N.Y.
Citronin	*Alternaria tenuis* *Aspergillus candidus* *Penicillium citrinin*		Div. of Bacteriology, Science Service, Ottawa
Clavacin	*Aspergillus clavatus* *Penicillium claviforme* *P. expansum* *P. patulum*	Herrick (11) Timonin (15)	Div. of Bacteriology, Science Service, Ottawa.
Gladiolic acid	*Penicillium gladioli*	Brian, Curtis, & Hemming (3)	Imperial Chemical Industries, Butterwick Res. Laboratories, England
Gliotoxin	*Gliocladium fimbriatum* *Trichoderma viride* *Aspergillus fumigatus*	Brian & Hemming (4) Weindling (20)	E. R. Squibb & Co., Brunswick, N.J.
Humulon	*Humulus lupulus*	Michener, Snell, & Jansen (14)	Div. of Bacteriology, Science Service, Ottawa
Lupulon	*Humulus lupulus*	Michener, Snell, & Jansen (14)	Div. of Bacteriology, Science Service, Ottawa
Viridin	*Trichoderma viride*	Brian & McGowan (5)	Imp. Chem. Ind., Butterwick Res. Labs., England

In Table II it can be seen that actidione, antibiotic XG, and viridin (pH 3.5) exerted a marked fungistatic effect on the germination of spores of *A. pisi* at concentrations above 1 p.p.m. Gliotoxin was fungistatic between 10 and 20 p.p.m., while clavacin, lupulon, and humulon were fungistatic at higher concentrations. Citronin showed no fungistatic activity at any of the dilutions used. At pH 3.5 viridin showed strong fungistatic activity but at pH 5.2 it was much less fungistatic. This is in accordance with the findings of Brian *et al.* (5). Gliotoxin, on the other hand, was unaffected by pH at the concentrations tested.

TABLE II

THE FUNGISTATIC ACTIVITY OF VARIOUS ANTIBIOTICS ON SPORE GERMINATION AND GROWTH OF *Ascochyta pisi*

Antibiotic	Dilution of antibiotic (p.p.m.)					
	0	1	10	20	50	100
Actidione	+	+	−	−	−	−
Antibiotic XG	+	+	−	−	−	−
Gladiolic acid	+	+	+	−	−	−
Gliotoxin (pH 3.5)	+	+	+	−	−	−
Gliotoxin (pH 5.2)	+	+	+	−	−	−
Humulon	+	+	+	+	−	−
Lupulon	+	+	+	+	−	−
Clavacin	+	+	+	+	+	−
Citronin	+	+	+	+	+	+
Viridin (pH 5.2)	+	+	+	+	+	−
Viridin (pH 3.5)	+	+	−	−	−	−

+ = *Growth visible.*
− = *No growth visible.*

II. Fungicidal Activity

In order to determine the fungicidal activity of the various antibiotics on spores of *A. pisi*, antibiotic dilutions were prepared aseptically in centrifuge tubes to which a spore suspension of 10,000 spores per ml. was added to give final antibiotic dilutions of 5, 15, 25, 50, and 100 p.p.m. The spores were allowed to remain in the antibiotics for 12 hr. at a temperature of 24° C. The tubes were then centrifuged and all but 0.5 ml. of the spore suspension was discarded. The spore suspension thus retained was washed and centrifuged twice until sterile conditions and finally 0.5 ml. was seeded on pea agar plates. Viable colony counts were made after five days. The results are presented in Table III.

TABLE III

THE FUNGICIDAL ACTIVITY OF VARIOUS ANTIBIOTICS ON SPORES OF *Ascochyta pisi*

Antibiotic	Time of exposure (hr.)	Dilution of antibiotic (p.p.m.)					
		0	5	15	25	50	100
Antibiotic XG	12	+	+	+	±	−	−
Gliotoxin	12	+	+	+	±	−	−
Actidione	12	+	+	+	+	−	−
Humulon	12	+	+	+	+	−	−
Gladiolic acid	12	+	+	+	+	−	−
Viridin	12	+	+	+	+	−	−
Clavacin	12	+	+	+	+	+	−
Lupulon	12	+	+	+	+	+	−
Citronin	12	+	+	+	+	+	−

+ = *Normal growth.*
± = *Only a trace of growth.*
− = *No growth.*

It will be seen in Table III, as might be expected, that the required fungicidal concentration of all antibiotics with a 12 hr. exposure is much higher than the fungistatic concentration (Table II) involving continuous exposure. Antibiotic XG and gliotoxin show greater fungicidal activity on the spores of *A. pisi* than do the other antibiotics tested: only a few spores remained viable after an exposure of 12 hr. at 25 p.p.m. All the antibiotics except lupulon, clavacin, and citronin were fungicidal with an exposure of 12 hr. at 50 p.p.m.

III. Phytotoxicity

(*a*) *Filter Paper Test*

Disease-free pea seed of Valley variety with a germinative capacity of 99% was used to determine the activity of the antibiotics on germination and on the developing seedling grown on filter paper. The filter papers, placed in sterile Petri dishes, were flooded with 10 ml. of the antibiotic at dilutions of 0, 1, 5, 15, 25, 50, and 100 p. p. m. Five pea seeds were placed in each dish. The plates were incubated at 24° C. for 10 days at the end of which time the peas were examined for germination and toxic symptoms on the seedlings. The results are summarized in Table IV.

TABLE IV

ACTIVITY OF ANTIBIOTICS ON PEA SEED GERMINATED ON FILTER PAPER

Antibiotic	Time of exposure (days)	Dilution of antibiotic (p.p.m.)						
		0	1	5	15	25	50	100
Actidione	10	+	×	×	×	×	−	−
Antibiotic XG	10	+	×	×	×	×	×	−
Viridin	10	+	+	×	×	×	×	−
Lupulon	10	+	+	×	×	×	×	−
Humulon	10	+	+	×	×	×	×	−
Gladiolic acid	10	+	+	×	×	×	×	−
Gliotoxin	10	+	+	×	×	×	×	×
Clavacin	10	+	+	+	×	×	×	×
Citronin	10	+	+	+	×	×	×	×

\+ = *Normal growth of seedlings.*
× = *Abnormal germination.*
− = *No germination.*

Phytotoxic symptoms of the antibiotics were noticed at relatively low concentrations. Table IV shows that actidione and antibiotic XG are toxic at a concentration as low as 1 p.p.m. Viridin, lupulon, humulon, gladiolic acid, and gliotoxin produced toxic symptoms at a dilution of 5 p.p.m. and clavacin and citronin at 15 p.p.m. It should be noted, however, that the original concentration of the antibiotic with which the filter paper was flooded would probably become increasingly higher during the 10 day germination period as the water evaporated.

(*b*) *Soil Test*

In order to determine the phytotoxicity of the antibiotics upon germination of pea seed grown in soil, 100-seed samples from the same source as used in the filter paper test were surface sterilized by treatment with a 2% chlorine solution for 15 min. and dried for 24 hr. on filter paper. The seed was then immersed in antibiotic dilutions of 25, 50, 75, and 100 p.p.m. for periods of 6, 12, 18, and 24 hr. After the specified period of time the seed was taken out of the antibiotic and placed on filter paper for one week to dry. When the seed was thoroughly dried it was sown in the greenhouse in sterilized soil at the rate of 100 seeds per flat. Emergence counts were taken two weeks after planting. The results are presented in Table V.

TABLE V

PERCENTAGE EMERGENCE OF VALLEY PEAS, SOAKED IN VARIOUS ANTIBIOTIC CONCENTRATIONS AND THEN PLANTED IN STERILIZED SOIL IN THE GREENHOUSE

Antibiotic	Period of treatment, hr.																			
	6					12					18					24				
	Antibiotic dilution (p.p.m.)																			
	0	25	50	75	100	0	25	50	75	100	0	25	50	75	100	0	25	50	75	100
Gladiolic acid	62	62	64	68	50	70	77	67	62	68	68	64	52	46	36	72	70	54	42	32
Viridin	80	68	70	76	72	74	72	76	54	50	82	68	71	58	53	84	78	65	59	54
Humulon	74	71	67	59	64	70	68	69	76	58	74	74	73	59	46	70	75	55	30	26
Gliotoxin	78	70	76	68	60	74	59	60	60	64	74	77	70	69	64	70	60	51	41	39
Antibiotic XG	79	86	79	71	35	83	74	53	26	12	78	75	46	15	10	83	79	40	9	4

As shown in Table V, the emergence of the seed treated for six hours at all dilutions up to 50 p.p.m. compared favorably with the check. However, at the 12 hr. treatment emergence was lowered, especially with the antibiotic XG treatment and to a lesser extent with viridin and humulon. With all the antibiotics tested, excepting gliotoxin, a soak of 18 hr. produced toxic symptoms as indicated by the lower emergence. At the 24 hr. soak, even at concentrations as low as 50 p.p.m., emergence with all treatments was considerably reduced.

IV. Effectiveness of Antibiotics in the Control of Internally Borne Ascochyta pisi in Pea Seed

A sample of Lincoln peas, known by previous plate test to contain 14% *Ascochyta pisi*, was treated in the same manner as in the previous test, i.e. surface sterilized with a 2% chlorine solution, air dried, immersed in the antibiotic solution for the specified time, dried again, then plated on pea agar in Petri plates at the rate of five seeds per plate. The plates were allowed to incubate for 10 days at the end of which time they were examined for germination of the seed and the number of seeds infected with *Ascochyta pisi*. The results are presented in Table VI.

TABLE VI

PERCENTAGE GERMINATION AND *Ascochyta pisi* IN NATURALLY INFESTED PEA SEED TREATED WITH VARIOUS ANTIBIOTICS

Antibiotic		Period of treatment, hr.																			
		6					12					18					24				
		Antibiotic dilution (p.p.m.)																			
		0	25	50	75	100	0	25	50	75	100	0	25	50	75	100	0	25	50	75	100
Gladiolic acid	Germination	72	92	78	74	80	82	86	76	82	78	68	70	82	88	74	82	62	58	80	68
	Ascochyta pisi	16	26	12	12	44	22	18	16	14	4	90*	18	20	28	32	8	6	88*	2	4
Viridin	Germination	84	80	74	84	66	74	68	80	66	84	44	59	68	62	78	70	82	80	76	66
	Ascochyta pisi	12	10	10	12	8	32	6	10	8	12	20	14	14	6	6	16	8	6	0	4
Humulon	Germination	84	72	86	72	84	76	84	72	82	68	84	74	76	88	76	74	76	84	56	56
	Ascochyta pisi	4	18	14	14	8	8	10	8	2	8	14	4	8	0	8	10	2	28	4	8
Gliotoxin	Germination	78	84	72	68	70	86	88	72	72	74	80	74	68	72	64	66	66	80	62	60
	Ascochyta pisi	22	16	10	10	12	10	4	4	14	8	12	8	6	6	4	68*	92*	6	10	8
XG	Germination	90	—	76	—	52	74	73	66	36	23	68	67	48	22	4	72	—	74	—	22
	Ascochyta pisi	14	—	8	—	10	13	12	9	5	4	11	0	1	0	1	8	—	0	—	0
Actidione	Germination	89	92	64	68	68	52	42	34	26	17	43	15	10	11	5	21	13	4	3	0
	Ascochyta pisi	7	5	0	5	7	10	4	0	1	3	4	1	0	0	0	7	0	1	0	0

* *The high percentage of A. pisi found in these cases can only be explained as failure of surface sterilization of the seed or spread of infection during treatment or possibly a combination of both.*

As seen in Table VI, the germination and degree of control of *Ascochyta pisi* varied considerably with the period of immersion in the antibiotic solution. None of the antibiotics tested gave any control in the six hour immersion. The pea seed treated for 12 hr. in actidione and antibiotic XG showed a marked reduction in the percentage of *A. pisi*. Actidione appeared to give good control at high concentrations after 18 hr. of treatment, but the germination was considerably reduced. With the exception of actidione and antibiotic XG, none of the other antibiotics tested gave adequate control of *A. pisi*, even after 24 hr. of treatment.

Although actidione and antibiotic XG were selected as the most promising on the basis of these tests, the results presented above suggest that gladiolic acid and viridin might be used successfully to control other diseases in which infection of the seed is of a more superficial type.

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TWO NEW SMUTS ON *CAREX* IN CANADA[1]

By D. B. O. Savile[2]

Abstract

Cintractia calderi is described from ovaries of *Carex backii*; it has been found in Manitoba and Ontario, and is characterized by very large spores with coarse, dark warts. The genus *Planetella* is erected to accommodate *P. lironis* found in the ovaries of *Carex maritima* in Keewatin and *C. leiophylla* in Yukon; the spores have thin, pale walls except for a dark brown, thickened equatorial ring, and the sori have a hard, prosenchyma coat.

Among numerous smuts collected in recent years in the ovaries of *Carex* spp. from all parts of Canada, two have been encountered that are conspicuously different from any previously described.

A species of *Cintractia*, found in phanerogamic collections of *Carex backii* from Manitoba and Ontario, has large rounded spores with deeply pigmented walls, ornamented with large warts. The only species of *Cintractia* on *Carex* with warts of comparable size are *C. subinclusa* and *C. caricis-oederi*; but in these species the warts are pale and flat-topped and the spores are small, whereas in the smut on *Carex backii* the warts are almost black and round-topped, and the spores are exceptionally large (Fig. 1).

Several species with large spores have been described, e.g., *Cintractia limosa*, *C. gigantissima*, and *C. liroi* with rounded spores, and *C. turfosa*, *C. irregularis*, and *C. pratensis* with spores that are moderately to strongly angular; but in all these species the spore walls vary from shallowly verrucose to almost smooth.

This species is named for Mr. J. A. Calder, of this Division. Mr. Calder's enthusiastic collecting of these smuts and painstaking checking of the hosts of many specimens is leading to an understanding of the genus *Cintractia* in Canada that was hitherto impossible.

Cintractia calderi sp. nov.

Sori fuliginosi, in ovariis. Teliosporae 21–32 × 19–24–(26)μ, compressae, ellipsoideae. Episporium ca. 1.0–1.5μ crass. verrucis exclusis, atrissimum; verrucis atrobrunneis, subcylindraceis, in apice rotundatis, ca. 0.5–1.5μ alt., 0.5–1.5μ lat. (raro usque ad 2.5μ long.), quorum centrum a centro abest 0.9–3.0μ.

Sori sooty, in ovaries. Teliospores 21–32 × 19–24–(26)μ, flattened-ellipsoid. Spore wall about 1.0–1.5μ thick excluding warts, very opaque black; warts dark brown, subcylindric, rounded above, about 0.5–1.5μ high, 0.5–1.5μ wide (occasionally to 2.5μ long), at 0.9–3.0μ spacing from center to center.

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Contribution No. 1106 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

[2] *Associate Plant Pathologist, Central Laboratory, Ottawa, Canada.*

In ovaries of *Carex backii* Boott: (DAOM 25881), Gillam, Man., 29 July 1950, leg. W. B. Schofield 1283 (TYPE); and (DAOM 25882) near Port Hope, Durham Co., Ont., 10 June 1950, leg. H. Reeve 365. Both specimens in the Mycological Herbarium, and the parent collections in the Phanerogamic Herbarium, Division of Botany and Plant Pathology, Ottawa, Canada.

The wall thickness and height of the warts can be determined only approximately in this species, because the wall is so heavily pigmented that even boiling in lactophenol will not clear it adequately. It should be noted that this species is inconspicuous because the perigynia of the sedge are largely enclosed in the long, sheathing bracts. Careful examination of specimens of *Carex backii* and the closely related *C. saximontana* may well yield further material of it.

On 9 July, 1950, the writer observed a smut in the overwintered inflorescence of *Carex maritima* Gunn. (*C. incurva* Lightf., not *C. maritima* O. F. Muell.) at Chesterfield Inlet, Keewatin. Although the sedge was abundant no other infected colonies were found. Some overwintered material was collected and the colony was marked. Further collections were made on 20 and 23 August. The sori of the current season were difficult to detect and would certainly have been overlooked had not the infection been noted in the spring. Close examination showed that many plants bore what seemed to be slightly swollen and darkened achenes, which, when broken open, yielded masses of dark spores. Later examination proved that what had appeared to be the achene coat was in fact composed entirely of fungus tissue. The achene is displaced by the sorus and the perigynium is split wide open. The spores are formed centripetally about a columella of host tissue heavily invaded by hyphae and surrounded by a dense layer of mycelium, as is usual in *Cintractia*. The columella may be connected apically with the remnants of the style. The sorus is covered by a firm coat consisting of an inner layer of more or less loosely matted mycelium and a hard, brown, prosenchymatous outer layer. This protective coat apparently remains intact until winter, for it showed no sign of rupturing on 23 August; in fact, it still partly invested the sori of the preceding year on 9 July. The broken coats of the overwintered sori were still quite hard and thick on 9 July and the manner of their opening is in doubt. Possibly they are split by frost or growth pressure; or some may survive for more than one winter and finally weather open. There is also a distinct possibility that many sori are broken or eaten by lemmings (*Lemmus trimucronatus* and *Dicrostonyx groenlandicus*), which might offer an important means of distribution, both by passage of spores through the intestinal tract and by sori being taken, like seeds, for storage in burrows.

This heavy coat is very different from the evanescent, white, papery, or arachnoid membrane surrounding the young sorus in typical species of *Cintractia*. The smut is also markedly distinct in the form of its spores from all species of *Cintractia* encountered by the author. Although the spores of the species of *Cintractia* vary greatly in size, sculpturing, and angularity, all tend to be flattened-ellipsoidal and all have a complete layer of pigmented

wall often with a partial, and rarely a complete, hyaline outer wall. In the smut on *Carex maritima* the spores are subspherical, and the wall is pale yellowish brown and thin, except for a deep chestnut, thickened, equatorial, inner band. This structure gives the randomly oriented spores a curious appearance, suggestive of little planets with dark equatorial zones and large polar ice caps (Fig. 2).

In 1949 Mr. J. M. Gillett collected *Carex leiophylla* Mack. at Carcross, Yukon, the type and only known locality of this sedge. In examining these plants, Mr. J. A. Calder noted hard-coated sori in the inflorescence and brought them to the writer's attention. This smut proved to be identical with that on *Carex maritima*. This situation introduces an interesting problem in the affinities of these two sedges, which are very similar in general appearance and which both grow in sand. On superficial examination *C. leiophylla* might well be taken for a robust plant of *C. maritima*. There are, however, several characters that caused Mackenzie (2) to put it in the section Atratae, rather than in the section Foetidae to which *C. maritima* is assigned. In the genus *Cintractia* there are instances of morphologically identical, but perhaps physiologically distinct, smuts occurring on hosts in different sections of *Carex*. Accordingly one must not place too much stress on the present finding. Nevertheless it seems a remarkable coincidence that this highly distinctive smut should have been found on two strikingly similar hosts, but not on others supposedly more closely related to either one than *C. maritima* and *C. leiophylla* are to each other.

It is felt that these distinctions in the form of the spores and the covering of the sorus merit the placing of this smut in a distinct genus. Unfortunately attempts to germinate the spores proved unsuccessful, and its affinities are therefore in doubt, but it is probable that it is related to *Cintractia*. It is proposed to erect for it a new genus *Planetella*, named in reference to the unusual spore form. The type species is named for the late J. I. Liro, whose studies in the Ustilaginales of Finland have been of great benefit to those studying these fungi in other countries.

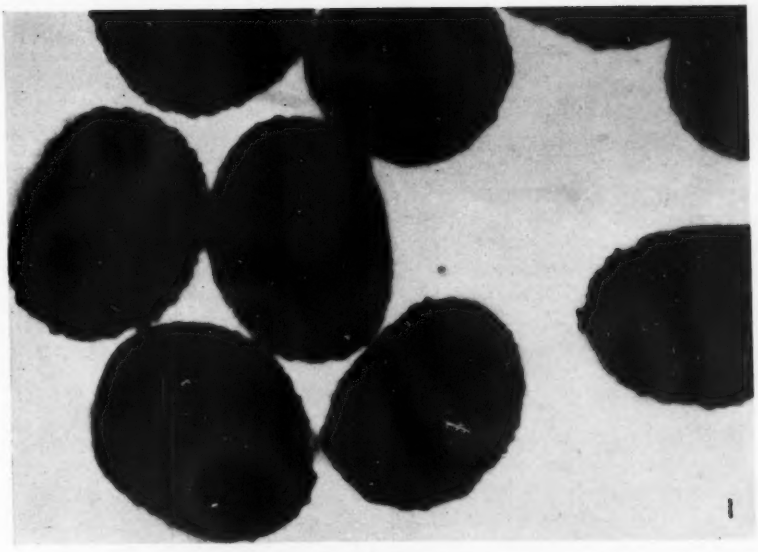
Planetella gen. nov. (Ustilaginales)

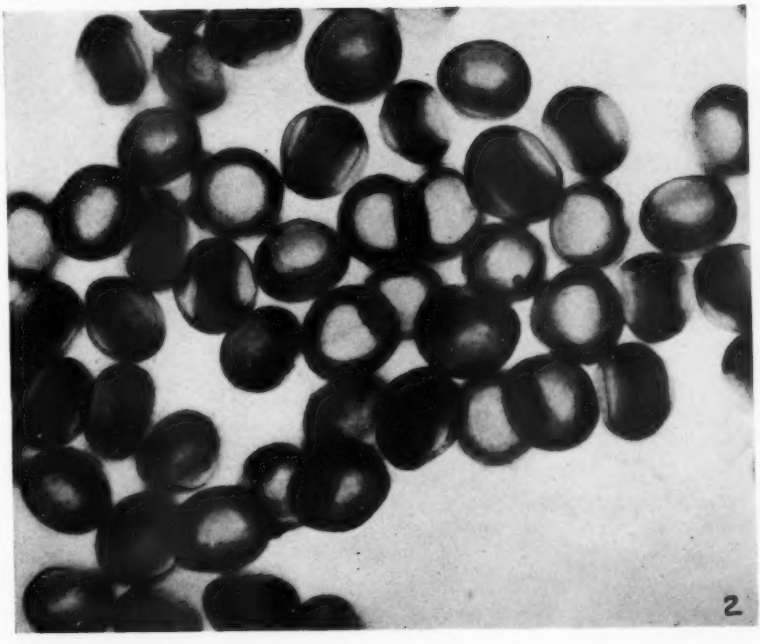
Sori in ovariis, crusta mycelii indurata instituti. Spori originant centripetalliter circa columellam centralem qua ex mycelio atque fragmentis hospitis generatur. Teliosporae subsphaeroideae; episporio tenui, pallido omnino nisi in annulo aequitoriali colore intento et interne incrassato.

FIG. 1. Spores of *Cintractia calderi*, ×1110, cleared in lactophenol; taken at equatorial focus; height of warts obscured by their close spacing.

FIG. 2. Spores of *Planetella lironis*, ×1110, cleared in lactophenol; spores in polar view appear as a dark ring; spores in equatorial view are crossed by a broad, central, dark band; spores in intermediate view appear asymmetrical.

PLATE I





Species typica, *Planetella lironis*.

Sori in ovaries, invested by a hard, mycelial coat. Spores formed centripetally about a central columella of mycelium and remnants of host tissue. Teliospores subspherical, with wall thin, pale except for an internally thickened, dark, equatorial band.

Planetella lironis sp. nov.

Sori crusta atrobrunnea instituti. Crusta externe dura et ex prosenchyma constat, ca. 20μ crass.; interne hyalina et ex laxiore texto mycelio, 10–30μ. Teliosporae diametro polari 9.5–13.5μ, diametro aequatoriali 10.0–15.0–(16.0)μ; si acervati atrae. Episporium ca. 0.3μ, pallide luteo-brunneum, minute verrucosum vel saepius subleve; verrucae si adsunt fere 0.2μ ambitu vel minores et spatio a centri ad centrum 0.5μ. Annulus aequatorialis intento-castaneus, 1.2–2.5μ crass., 5.0–7.5μ lat.

Sori invested by a dark brown coat with an outer layer of hard, dark, prosenchyma tissue about 20μ thick, and an inner layer of hyaline, looser mycelium 10–30μ thick. Teliospores 9.5–13.5μ polar diameter, 10.0–15.0–(16.0)μ equatorial diameter; black in mass. Wall *ca.* 0.3μ thick, pale yellowish brown, minutely verrucose or often apparently smooth, the warts less than 0.2μ in height and width and at intervals of about 0.5μ; with an internal, dark chestnut, equatorial band 1.2–2.5μ thick $\times$ 5.0–7.5μ wide.

In ovaries of *Carex maritima* Gunn. (DAOM 25883), Chesterfield Inlet (63°21′ N, 90°42′W), Keewatin, 23 Aug. 1950, leg. D.B.O. Savile 1575 and C. T. Watts (TYPE); on *C. maritima* (DAOM 25885), Chesterfield Inlet, 9 July 1950, leg. Savile 921 and Watts, and (DAOM 25886), Chesterfield Inlet, 20 Aug. 1950, leg. Savile 1559 and Watts; on *C. leiophylla* Mack. (DAOM 25884), Carcross, Yukon, leg. J. M. Gillett 3772. Type in the Mycological Herbarium, Division of Botany and Plant Pathology, Ottawa, Canada. Co-type material is being distributed to: University of California, Berkeley; Harvard University; University of Michigan; University of Toronto; University of Wisconsin; State College of Washington; Division of Mycology and Disease Survey, Beltsville, Md.; Commonwealth Mycological Institute; Naturhistoriska Riksmuseet, Stockholm; Botanical Museum, Oslo; Lund Botanical Exchange Club.

There is a possibility that this smut has been collected once previously. Allescher and Hennings (1) report the occurrence of *Cintractia caricis* on *C. incurva* (*C. maritima*) from the Umanak district in west Greenland. The writer has not been able to locate this specimen, which is not at the Botanical Museum, University of Copenhagen. Possibly the collection was a small one and was all sent to Allescher, in which case it was presumably destroyed during the second world war. There is no material on this host at the Botanical Museum, Oslo. It does not seem likely that the present smut would have been placed in *Cintractia caricis*, despite the diverse forms that have been included in that species by some writers; and it is probable that a second smut actually occurs on *Carex maritima*.

Acknowledgments

The writer wishes to express his appreciation to Dr. I. Jørstad and Prof. N. F. Buchwald, for searching for smuts on *Carex maritima* in the collections at Oslo and Copenhagen, respectively. His thanks are also due to Dr. B. Boivin, of this Division, for assistance in preparing the Latin diagnoses, and to Dr. George W. Fischer, State College of Washington, for reading the manuscript.

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UPTAKE OF P^{32} BY SOME CEREALS[1]

By J. M. Naylor,[2] G. W. R. Walker,[3] D. Murray,[4] C. O. Person,[5] J. W. T. Spinks,[6] and T. J. Arnason[7]

Abstract

When wheat grains were germinated individually in test tubes containing 0.1 ml. solution in which initially 6.5-10 mrd. P^{32} in the form of phosphate were included, over 90% of the activity was absorbed by the grain and seedling within 13 days. At five days over 80% of the absorbed activity was in the embryo with less than 20% in the grain coats and endosperm. When plants were grown in washed sand of high quartz content about 5% of the activity added in solution to the sand at 31 days or at 40 days was absorbed by the plants. Activity added to soil in which barley plants were growing resulted in activity absorptions ranging from 0.97 to 10.4% of the amounts supplied. More P^{32} was absorbed when carrier P was provided than when carrier-free solutions were used. A method permitting uptake measurements to be made at intervals with the same plants was also tried. Initially, 1.66 mrd. P^{32} were available to each plant. At intervals, determinations of uptake were made. Then P^{32} was added to the solution to bring the level up to 1.66 mrd. per plant. The total energy received by individual *vulgare* wheat plants from decay of absorbed P^{32} was calculated to be 54.6 gram-roentgens.

Introduction

The use of radio elements for mutation production is still in its infancy and methods of applying the isotopes for maximal mutation effects are not yet standardized. Reported methods that have been used with some success are: germinating seeds in radioactive solutions (1, 7), injection of P^{32} into plants (1), placing cut stems in radioactive solutions (8), adding P^{32} to food medium of *Drosophila* larvae (3, 4, 5), and supplying P^{32} in the food of adult flies (9).

The present report deals with methods that have been used to introduce P^{32} into wheat and barley plants with a view to use for mutation-induction. These include (1) germinating grains and growing seedlings individually in test tubes in radioactive solutions for 3-13 days, then transferring to untreated soil; (2) adding P^{32} to sand or soil in which plants are rooted, with single or multiple applications of P^{32}; (3) growing plants to the microsporocyte stage in solutions with measured amounts of P^{32} provided, thus allowing for continuous uptake of P^{32}. The amounts of activity used are recorded in milli-rutherfords (mrd.). One mrd. equals 1000 disintegrations per second.

P^{32} Uptake by Seedlings Growing in Solutions Containing Radiophosphorus

Several small experiments have been performed to determine the rates at which P^{32} is absorbed from measured amounts of solution by the seedlings.

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Contribution from the University of Saskatchewan, Saskatoon, Sask.
[2, 3, 4, 5] *Graduate Students.*
[6] *Professor of Physical Chemistry.*
[7] *Associate Professor of Biology.*

The information gained is of value in indicating how long seedlings should be left in solution before transferring to soil. Two such experiments are detailed below:

One hundred grains of Thatcher wheat (*Triticum vulgare* Vill.) were germinated individually in test tubes containing initially 8.25 mrd. of carrier-free P^{32} in the form of H_3PO_4 present in 0.1 ml. Knop's solution. Prior to the addition of the activity the Knop's solution was P-deficient. At 12.5 hr. 20 seeds were removed, 10 of these were weighed and their P^{32} content determined by counting with a Geiger–Müller counter. The remaining 10 seeds were dissected with needles into embryo, grain coat, and endosperm. These were air-dried, weighed, and the P^{32} content was determined. Similar determinations were made at 50, 100, and 125 hr. The results, averaged, are presented in Table I and illustrated graphically in Fig. 1.

TABLE I

THE AVERAGE WEIGHT IN MILLIGRAMS AND THE AVERAGE P^{32} CONTENT OF *Triticum vulgare* GRAINS GERMINATED IN 0.1 ML. SOLUTION CONTAINING INITIALLY 8.25 MRD., 12.5-125 HR. AFTER THE BEGINNING OF GERMINATION

Hours	Whole grains*		Dissected grains*						Total dissect.		% absorbed P^{32} in embryo
			Embryo		Endosperm		Grain coats				
	Weight mgm.	Activity, mrd.	Wt. mgm.	Act., mrd.	Wt. mgm.	Act., mrd.	Wt. mgm.	Act., mrd.	Wt. mgm.	Act., mrd.	
12.5	34.2	0.0554	1.00	0.00584	19.3	0.00452	11.64	0.0421	31.94	0.05246	11.1
25	34.7	0.149	0.90	0.0385	26.5	0.0124	6.77	0.0599	34.17	0.1108	34.7
50	34.2	0.378	1.06	0.414	27.4	0.0310	6.09	0.0945	34.55	0.5395	76.7
100	30.3	2.21	3.22	2.03	18.3	0.0960	6.30	0.1232	27.82	2.2492	90.3
125	30.0	2.61	8.02	2.24	20.8	0.0176	6.48	0.0836	35.30	2.3412	95.7

* *Average, for 10 grains.*

The amount of P^{32} absorbed in the first two days was only 5% of the total available. In the next three days uptake was rapid. Over 30% of the available activity was in the grain and seedling at 125 hr. Between 50 and 100 hr. absorption of P^{32} was most rapid, activity content rising from 0.38 mrd. to 2.21 mrd. per counted whole grain. The highest observed P^{32} concentration in embryos, 0.63 mrd. per mgm., occurred at 100 hr. After 100 hr. growth was relatively more rapid than was P^{32} uptake. At 125 hr. P^{32} concentration in embryos had dropped to 279 mrd. per gram.

In a second test *T. vulgare* wheat grains were germinated individually in test tubes containing, initially, 10.8 mrd. P^{32} with carrier in 0.1 ml. solution. The level of the liquid was maintained by adding drops of Knop's solution as required. Grains were removed in groups of 20 at 24-hr. intervals beginning at 48 hr. The embryo (or seedling) was dissected out of each grain so that the proportions of the total absorbed activity in the embryo and the remainder of the grain might be determined. The results are recorded in Table II.

At the end of seven days slightly less than one-half of the available activity had been absorbed by the germinating grains. The embryo's share of the absorbed P^{32} rose from 73.1% on the third day to 88.1% on the seventh day.

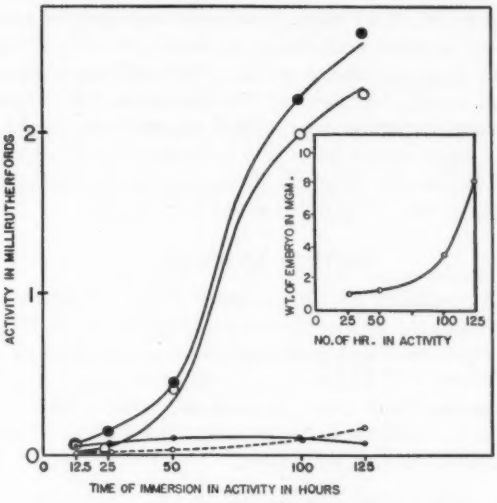

FIG. 1. Mean absorption of P^{32} activity per wheat grain, embryo, endosperm, and grain coat from solution containing, initially, for each grain, 8.25 mrd. Symbols ●, whole grain; ○, embryo; ——●——, grain coats; ——○——, endosperm. At right, embryo weight at 25, 50, 100, and 152 hr. plotted separately.

TABLE II

UPTAKE OF P^{32} BY EMBRYOS COMPARED WITH UPTAKE BY THE GRAIN COATS AND ENDOSPERMS OF GERMINATING WHEAT GRAINS

Time in days	Total P^{32} per tube, mrd.	Mrd. P^{32} absorbed by			% of P^{32} absorbed	% absorbed P^{32} in	
		Whole grain*, including embryo or seedling	Grain* coats and endosperm	Embryo* (young plant)		Grain coats and endosperm	Embryo (young plant)
2	9.32	—	—	1.43	—	—	—
3	8.87	3.05	0.82	2.23	34.4	26.9	73.1
4	8.45	3.72	0.74	2.98	44.0	19.9	80.1
5	8.04	3.45	0.54	2.91	42.9	15.7	84.3
6	7.65	3.58	0.33	3.25	46.8	9.2	90.8
7	7.28	3.54	0.42	3.12	48.6	11.9	88.1

* *Average, for 20 grains.*

It is apparent that under the conditions prevailing in these investigations most of the absorbed P^{32} passes to the young growing plants with a small proportion only being held in the grain coats and endosperm.

The amount of activity passing from solution to seeds and seedlings was determined at the end of 14 days. Seedlings were started in separate test tubes with 0.1 ml. solution containing 13, 26, or 39 mrd. P^{32} in the form of H_3PO_4. The pH of the solution was 6-8. Ten seedlings were grown in each of the activity amounts. As the free liquid disappeared one or more drops of P-deficient Knop's solution were added as required; at 14 days counts made on activity remaining in test tubes indicated that 79.4 - 93.7% of the P^{32} had entered the plants. The % absorption of P^{32} was nearly the same at all three concentrations of activity which were tested.

Multiple Treatment

Since there is a possibility that the number of recoverable mutations would be increased by repeated application of sublethal doses of P^{32} during plant development an experiment was performed to provide preliminary information on this point. Wheat and barley plants were given one, two, or three doses of P^{32}, the applications being made (*a*) at 24-hr. seedling stage (*b*) at 31 days, and (*c*) at 40 days. Microsporocytes were developing or were about to develop at the time of the last treatment.

The seeds were germinated individually in test tubes containing 0.1 ml. solution. At 13 days the seedlings were transferred to crocks containing washed sand of high quartz content. Essential minerals were supplied in normal Knop's solution which was provided at intervals of two days to the plants growing in sand. Plants were supplied with P^{32} as follows: 6.5-0-0; 6.5-0-65; 6.5-6.5-65; 0-0-6.5; 0-0-65. The figures refer to mrd. P^{32} supplied at 24 hr., 31 days, and 40 days, respectively. When near maturity and at least one week after the last application of P^{32}, 10 plants were wet-ashed and the activity content determined. When allowance had been made for decay the figures obtained indicated that 31% of the P^{32} supplied at 24 hr., 5% of that supplied at 31 days, and 5.6% of that supplied at 40 days was in the plants at the time of harvesting. Wheat plants receiving the heaviest dosage of P^{32} showed some wilting and yellowing of leaves after the last application of activity. Two plants died and several others failed to form seeds.

Although only a small proportion of the activity added to the sand in which plants were rooted was absorbed, observations on chromosome aberrations in anthers, and on morphological mutants in later generations indicate that these "late" treatments are useful in increasing the numbers of viable chromosome aberrations and visible mutations.

P^{32} Absorption from Treated Soil

With mutation-induction as the main object 2500 barley plants (*Hordeum vulgare* L. variety Montcalm) were treated with radiophosphorus. P^{32}, in

the form of H_3PO_4 solution (5-15 ml.), was poured into soil around seeds and growing plants. One late application was made to roots below the crown level. The crown roots were exposed by removing soil. After each application of activity plants were well watered. Carrier P was supplied at the rate of 0.02 gm. of $NH_4H_2PO_4$ per ml. of active solution except where otherwise specified.

Whole treated plants were harvested at intervals after treatment. These were wet-ashed and activity content determined with a dip counter.* A summary of treatments given and amounts of P^{32} found in counted plants is provided in Table III. The lots designated A, A′, B, and B′ were grown in soil in pots. All others were treated in the field.

TABLE III

P^{32} UPTAKE FROM TREATED SOIL BY BARLEY PLANTS

Lot	Seeding date	Mrd. P^{32} added to soil*			Collection date	No. of plants counted	Mrd. plant**	% supplied activity in plant
		Around seed	At surface, June 14	Below crown roots, June 29				
A	Oct. 30	40	—	—	Nov. 10	10	0.52	2.2
A′	Oct. 30	40	—	—	Nov. 10	10	0.23	0.97
B	Oct. 30	40	—	—	Nov. 20	7	0.39	2.7
B′	Oct. 30	40	—	—	Nov. 20	8	0.24	1.6
C	May 11	111	585	—	June 24	10	5.30	1.5
D	May 15	—	—	1618	July 5	5	58.00	4.9
E	May 11	111	585	1618	July 5	5	146.40	10.4

* *Carrier P included in all treatments except for lots A′, B′, and the June 14 application.*
** *Calculated to date of collection.*

From the results it appears that surface application to the soil is less effective than application below crown-root level. Greater absorption of activity occurred when carrier P was provided than when carrier-free P^{32} was used.

Continuous Uptake of P^{32}

An attempt was made to establish an accurate technique for determining the absorption of P^{32} by cereals throughout the growing season. In previous experiments dealing with the effects of P^{32} in cereals, a major problem has been the exact determination of the amount absorbed (1, 2, 6). Generally, the rate of absorption has been determined statistically by ashing a number of the treated plants, and quantitatively measuring the amount of radioactive phosphorus in the residue by means of a Geiger–Müller counting chamber. Where a cytological study of the treated plants is intended or where a maximum yield of mutant offspring is desired, this sacrifice of plants is often

* *We are indebted to Dr. A. M. Kristjanson for these determinations.*

inconvenient. This difficulty becomes serious in mutation experiments where P^{32} is applied at various stages of growth since quantitative determinations following each treatment are generally desirable.

The method of culture described here was designed to avoid the above difficulties. It also permits the maintenance of known amounts of P^{32} within the plants over extended periods in the growing season.

Materials and Methods

Grains of *Triticum vulgare* Vill., *T. durum* Desf., and *T. monococcum* L. were surface sterilized and germinated on blotters for 48 hr. At the end of this time six vigorous seedlings of each kind were transferred individually to test tubes each containing 1.66 mrd. P^{32} in 0.1 ml. of solution. The pH of the solution was between 5 and 6. Roots only were immersed in the solutions. The level of the liquid was kept fairly constant by adding Knop's nutrient solution as required. Beginning at the fifth day the level was raised by adding 2 ml. Knop's solution to each test tube.

At 14 days, seedlings were transferred to a glass tank containing 25 liters of Knop's solution. The plants were supported on a perforated aluminum plate placed at the surface of the liquid. One root of each plant was guided

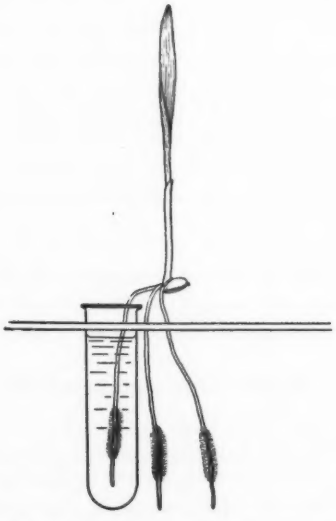

FIG. 2. Wheat seedling with one root in a test tube containing P^{32}; other roots in inactive nutrient solution.

into a 10 ml. test tube containing initially 1.66 mrd. P^{32}, the remaining roots were suspended in the tank solution (Fig. 2). As the plants grew it became progressively more difficult to manipulate the roots. The perforated plate

was then replaced by a strip of aluminum $\frac{3}{4}$ in. wide and 1/16 in. thick. The test tubes were attached to the metal strip by rubber bands at 3-in. intervals. With this arrangement control over the positions of plant parts became relatively easy.

The crown of each plant was kept at the surface level of the tank solution. By attaching leaves to horizontal wires the shoots were kept erect. The nutrient solution was aerated for 20 min. each day by means of a pressure-bottle attached to an aspirator. The solution was changed at 10-day intervals.

P^{32} uptake by plants was measured indirectly by determining, at intervals, the amounts still remaining in the test tubes. After each such determination P^{32} was added to bring the amount in each tube to 1.66 mrd. The proportion of the P^{32} passing from the test tubes to the plants was calculated for each period. On the assumption that absorbed P^{32} was retained within the plants, calculations were also made respecting the amount of energy released in tissues by the decay of the radioactive material.

P^{32} was continuously available to the plants for 66 days. During this period, six treatments were administered (Table IV). At the end of the final

TABLE IV

UPTAKE OF P^{32} (EXPRESSED IN DISINTEGRATIONS PER MIN. $\times 10^{-4}$) BY INDIVIDUAL PLANTS DURING SIX PERIODS OF TREATMENT

	Treatment					
	1	2	3	4	5	6
	Days following germination					
	2-16*	16-25	25-41	41-50	50-58	58-68
T. vulgare, Plants 1-6	4.5	3.5	4.0	3.7	2.7	2.4
	4.5	3.1	4.2	3.6	5.1	2.6
	4.5	3.6	3.4	3.9	4.0	3.2
	4.5	4.6	4.9	3.1	4.5	1.1
	4.5	4.7	4.0	4.6	3.1	2.3
	4.5	5.0	4.4	4.0	3.9	2.4
Mean ± S.D.**		4.1 ± 0.7	4.2 ± 0.5	3.8 ± 0.5	3.9 ± 0.8	2.3 ± 0.6
Mean (mrd.) ± S.D.		0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.4 ± 0.1

* *Mean values only, determined from 20 seedlings.*
** *Standard deviation.*

treatment, all plants were transplanted into soil. All plants with the exception of one Einkorn survived the six treatments and eventually matured. The one casualty may have died as a result of injury during transplanting. While the first tiller produced by each plant set no seed, second tillers were generally at least partially fertile.

Results

The rates of P^{32} uptake were very similar in the three species. For illustration purposes the complete data relating to absorption by the *T. vulgare* plants will suffice. Summaries of the results pertaining to the other two species are provided. In Table IV the uptake of P^{32} by individual *vulgare* plants in each of the six treatment periods is recorded. The total P^{32} content of plants at the various sampling dates is given in Table V. The percentage of the P^{32} provided which was absorbed is recorded separately for each treatment-period in Table VI.

TABLE V

ACTUAL DISINTEGRATIONS OF P^{32} ($\times 10^{-4}$ PER MIN.) IN PLANTS AT SIX DATES PRIOR TO FLOWERING

	Treatment					
	1	2	3	4	5	6
	Days following germination					
	16	25	41	50	58	68
T. vulgare, Plants 1-6	4.5*	6.5	7.1	8.4	8.5	7.7
	"	6.1	7.1	8.3	10.8	9.4
	"	6.6	6.5	8.2	9.6	9.2
	"	7.6	8.5	8.7	10.5	7.7
	"	7.7	7.6	9.6	9.7	8.4
	"	8.0	8.2	9.4	10.4	8.9
Mean ± S.D.**		7.1 ± 0.7	7.5 ± 0.7	8.8 ± 0.6	9.9 ± 0.7	8.6 ± 0.9
Mean (mrd.) ± S.D.		1.2 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.2

* *Mean value only, determined from 200 seedlings.*
** *Standard deviation.*

TABLE VI

PERCENTAGE UPTAKE OF P^{32} (BASE 1.66 MRD.) DURING THE SIX PERIODS OF TREATMENT, ± STANDARD DEVIATION

	Treatment					
	1	2	3	4	5	6
T. vulgare	95.7	62.1 ±10.6	63.6 ±7.6	57.6 ±7.6	56.5 ±11.6	36.5 ±9.5
T. monococcum	97.9	56.1 ±16.6	60.0 ±9.0	71.2 ±13.6	73.9 ±4.3	27.0 ±19.0
T. durum	97.9	51.5 ±18.1	54.5 ±16.6	78.8 ±7.6	78.3 ±7.2	42.9 ±20.6

The data in Table V can be used to determine the energy liberated in the tissues of these plants during their period of growth. If the mean values shown in the table are plotted, the resulting curve (Fig. 3) indicates the rates

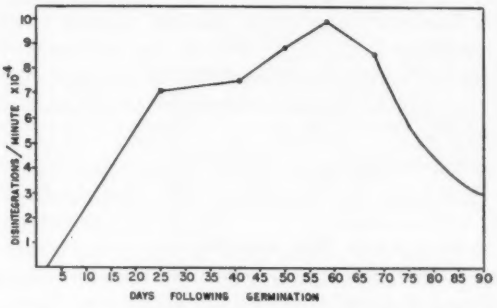

FIG. 3. Rates of disintegration of P^{32} contained in wheat plants at successive stages of growth

of disintegration of P^{32} per plant during the experiment. By calculating the area under the curve, total disintegrations, hence energy liberated in the tissues can be calculated. The total number of disintegrations per plant in each group is:

T. vulgare : 6.34×10^9
T. durum : 6.55×10^9
T. monococcum : 6.46×10^9

If one half of the beta particles emitted through disintegration of P^{32} atoms were stopped in the cells, the following amounts of energy were absorbed by each plant:

T. vulgare : 2.20×10^{15} ev. = 42.2 gram-roentgens*
T. durum : 2.28×10^{15} ev. = 43.6 "
T. monococcum : 2.25×10^{15} ev. = 42.9 "

The figures given above apply to the 66 days between the initial treatment and the final sampling. The plants did not flower until about three weeks later. The disintegrations occurring within the plant during this period must therefore be considered potentially effective in the production of mutant gametes. The energy liberated during the 21 days after the final sampling date increased the effective radiation to the following values:

T. vulgare : 2.85×10^{15} ev. = 54.6 gram-roentgens
T. durum : 2.97×10^{15} ev. = 56.7 "
T. monococcum : 2.90×10^{15} ev. = 55.3 "

* *A gram-roentgen is the energy absorbed when a dose of one roentgen is given to one gram. In air 1 gm-r. = 84 ergs.*

Discussion

Since the sampling dates were rather far apart it may be that decay of P^{32} in plants was proceeding faster than absorption for a part of the period. Maximal levels of P^{32} in the plants would not then occur at the sampling times. Since no determinations of P^{32} loss to the nutrient solution in the tank were made, there is a possibility that a slight error in the estimated P^{32} content of plants results. Moore (10), has established that there is a small movement of phosphorus from roots of corn plants to the nutrient solution when plants are grown in liquid culture.

By use of the method outlined here it is possible to maintain a fairly constant but low intensity of radiation over an extended time period. In work designed to produce viable useful mutations, low intensity, long continued may be preferable to the short exposure, high intensity treatments commonly used in the past. With low radiation intensity, gene mutations and single breaks are favored at the expense of more complex rearrangements. Since gene mutations have usually no effects on chromosome behavior sterility effects of mutations may be relatively small.

Acknowledgment

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EFFECT OF PASMO DISEASE OF FLAX ON THE YIELD AND QUALITY OF LINSEED OIL[1]

By W. E. Sackston[2] and R. B. Carson[3]

Abstract

Heavy infections of pasmo significantly reduced the oil content of flaxseed and the iodine number of the oil. The effects of pasmo infection on oil content and iodine number of the oil were similar to those caused by a hot, dry climate. Oil content was affected similarly by pasmo infection and by flax rust infection, but the two diseases had dissimilar effects on iodine number of the oil. Greatest reductions in oil content and iodine number resulted from pasmo inoculations made when the flax plants were flowering. Inoculations made when the seed was ripening had little or no effect on yield and quality of linseed oil.

Introduction

Flax is grown in Western Canada primarily for the linseed oil that is extracted from the seed. The acreage sown to flax is determined largely by cash returns to the growers, many of whom prefer wheat as a cash crop. When the price of flax is high because of a heavy demand for linseed oil for industrial or military purposes, the acreage of flax may increase rapidly; when the price of flax goes down, the acreage of flax may decrease even more rapidly (11).

Returns to the flax grower are affected by the yields per acre, as well as by the price per bushel. The yield of flaxseed may be reduced by diseases. The effect of pasmo disease, caused by *Septoria linicola* (Speg.) Gar., on the seed yield of four flax varieties grown in experimental plots was investigated. It was found that heavy infections of pasmo caused premature ripening and reduced the seed yield and weight per thousand kernels of all four varieties (11). This paper reports the effect of pasmo disease on the oil content of the seed and the iodine number of the oil of flax grown in inoculated plots.

Methods

Four flax varieties, Crystal, Viking, Royal, and Redwing, were grown in replicated plots at Winnipeg and Morden in the three years 1945 to 1947. The new, rust-resistant, yellow-seeded varieties Crystal and Viking were chosen because they were, respectively, among the least susceptible and most susceptible to pasmo. The brown-seeded varieties Royal and Redwing were included because they were the standard flax varieties throughout much of Western Canada. Plots of each variety were inoculated in 1945 by spreading pasmo-infected flax straw between the rows or by spraying the plants with spore suspensions of *S. linicola*, at two stages of plant growth. Spray inoculations were made at five dates in 1946 and four dates in 1947.

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Contribution No. 1095, Division of Botany and Plant Pathology, Science Service, Canada Department of Agriculture, and Contribution No. 197, Division of Chemistry, Science Service, Canada Department of Agriculture.

[2] *Plant Pathologist, Dominion Laboratory of Plant Pathology, Winnipeg, Canada.*

[3] *Chemist, Division of Chemistry, Science Service, Ottawa, Canada.*

Disease readings and yield data were obtained at Winnipeg and Morden in 1945. The plots at Winnipeg were discarded in 1946 because of poor, uneven, germination, and the plots at Morden were discarded in 1947 because a heavy rainstorm several days before harvest stripped most of the bolls from inoculated flax plants. Thus, although plots were seeded at two stations in each of three years, data were obtained for only four "station-years".

The center two rows of each four-row plot were harvested, dried, and threshed with a Kemp rod-row thresher (7) with the air-blast shut off so that no seed was lost. The seed was cleaned carefully with sieves and a small fan, to make sure that even the light seed was retained. Thousand-kernel weights were determined for samples from all plots. Samples of seed from each plot were submitted to the Plant Chemistry Unit, Division of Chemistry, Science Service, Department of Agriculture, Ottawa, where the oil content and iodine number of the oil of each sample were determined under the direction of the junior author. All data were analyzed statistically at Winnipeg.

The method used to determine oil content was essentially that of the American Oil Chemists' Society. A 1 gm. sample of ground seed was extracted for two hours in the Goldfisch apparatus with Skellysolve F. It was then transferred to a mortar and ground for one minute with sand. After a further two-hour extraction period the solvent was removed by evaporation and the oil was dried to constant weight at 100° C.

The iodine number was determined on oil pressed from the whole seed with a Carver press. The short form of the Wijs method developed by Hiscox (5) was used.

Results

The effects of the various inoculations with pasmo on disease development, yield of seed, and thousand-kernel weight of the four flax varieties were reported in detail in an earlier paper by the senior author (11).

The differences in the oil content of the seed due to inoculating flax plots at various intervals after seeding were highly significant statistically in all four station-years. Seed from uninoculated check plots yielded the highest percentage of oil in three station-years, although these values were not significantly different from the next highest percentage in any case. In the fourth station-year (Morden, 1946), seed from plots sprayed in the seedling stage had the highest oil content, although the percentage was not significantly higher than that from three other treatments. The oil content values for plots sprayed at the flowering stage were very significantly lower than those for all other treatments in three station-years. In the fourth station-year (Morden, 1946), seed from plots sprayed at flowering time contained the lowest percentage of oil, but the mean value was not significantly different from that for plots sprayed at the boll-filling stage. The difference between the mean oil content of plots sprayed at the flowering stage and plots sprayed at the seedling, post-flowering, and ripening stages at Morden in 1946 exceeded the 1% level of significance, and almost reached the 1% level for uninoculated check plots.

In three station-years the oil content of seed from plots sprayed at the seedling stage was very significantly higher than from plots sprayed at the flowering stage, and very significantly lower than from plots sprayed late in the season and from uninoculated check plots. In the fourth station-year (Morden, 1946), seed from plots sprayed at the seedling stage had the highest oil content. Inoculations made when the plots were ripening in 1946 and 1947 had no significant effect on the oil content of the seed. Mean values for each date of inoculation are shown in Fig. 1.

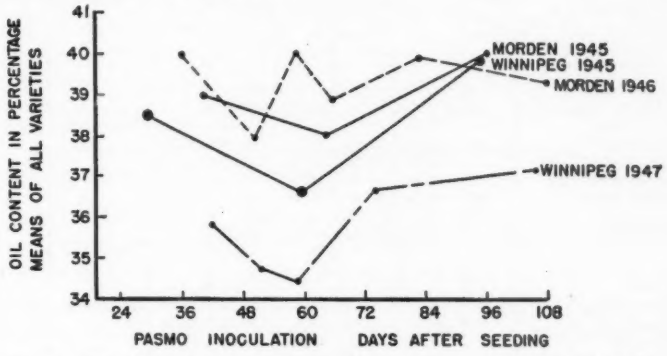

FIG. 1. Effect of date of inoculation with pasmo on oil content of flax in four station-years.

The extent of reduction in oil content due to inoculation with pasmo is indicated in Table I, in which the mean oil content of seed from all plots

TABLE I

EFFECT OF PASMO INFECTION ON THE OIL CONTENT OF FLAXSEED FROM INOCULATED PLOTS

Station	Year	Inoculation (days after seeding)	Oil content as percentage of uninoculated check
Morden	1945	39	96.83**
		64	94.66**
Winnipeg	1945	30	96.81**
		59	91.82**
Morden	1946	36	101.57
		51	97.40*
Winnipeg	1947	40	96.53**
		52	93.13**

** Reduction from check almost significant at the 5% level. Difference between values for seedling and flowering inoculations significant beyond the 1% point.*

*** Reduction from check significant beyond the 1% point. Difference between values for seedling and flowering inoculations significant beyond the 1% point.*

inoculated at the seedling and flowering stages respectively is given as a percentage of the oil content of seed from uninoculated check plots. The mean oil content data for all tests are given in Table II.

TABLE II

OIL CONTENT OF FLAXSEED IN PER CENT. MEANS OF ALL REPLICATES

Inoculated	Days after seeding	Crystal	Viking	Royal	Redwing	Treatment mean	Minimum significant difference between means		
								5%	1%
Morden, 1945									
Flowering—spray	64	38.62	38.63	38.30	37.32	38.22	Treatment	.654	.905
Seedling—spray	39	39.62	38.42	39.72	38.62	39.10			
Seedling—straw		40.50	40.04	40.46	39.38	40.09			
Seedling—chopped straw		40.46	39.40	41.24	38.98	40.02	Variety	.252	.336
Uninoculated check	96	39.74	41.38	41.84	39.06	40.38			
Variety mean		39.85	39.84	40.38	38.75				
Winnipeg, 1945									
Flowering—spray	59	38.04	34.90	37.07	36.39	36.60	Treatment	.759	1.051
Seedling—spray	30	39.09	38.90	38.32	38.06	38.59			
Seedling—straw		39.42	40.07	38.82	38.26	39.10			
Seedling—chopped straw		39.62	40.54	39.62	38.48	39.57	Variety	.396	.528
Uninoculated check	94	39.49	40.89	40.62	38.45	39.86			
Variety mean		39.22	39.45	39.18	37.99				
Morden, 1946									
Flowering—spray	51	39.90	38.30	37.60	37.40	38.32	Treatment	.831	1.151
Seedling—spray	36	40.20	40.00	40.20	39.40	39.96			
Postflowering—spray	58	40.00	40.60	40.30	38.80	39.91			
Boll filling—spray	65	39.50	39.80	39.60	36.20	38.78			
Ripening—spray	83	39.50	40.70	40.40	39.00	39.90	Variety	.557	.744
Uninoculated check	108	38.30	40.00	40.40	38.80	39.34			
Variety mean		39.49	39.84	39.69	38.21				
Winnipeg, 1947									
Flowering—spray	52	36.69	36.49	30.98	34.30	34.62	Treatment	.712	.986
Flowering—spray	58	35.69	35.33	32.45	34.00	34.37			
Seedling—spray	40	37.14	35.90	35.38	35.11	35.88			
Flowering—spray (1/100)		35.52	35.85	35.28	36.71	35.84			
Ripening—spray	74	37.05	38.18	35.65	36.04	36.73	Variety	.780	1.042
Uninoculated check	105	37.56	38.07	36.27	37.03	37.17			
Variety mean		36.56	36.63	34.33	35.53				

The relationship between oil content of flaxseed and pasmo infection was studied further by calculating correlation coefficients (Table III) from the data for each variety at each station. The negative correlations for all data except those for Crystal and Redwing at Morden in 1946 indicate that pasmo generally affects oil content adversely. Uniformly high disease ratings on

TABLE III

CORRELATION COEFFICIENTS: PASMO DISEASE RATING AND OIL CONTENT OF FLAXSEED

Variety	Morden 1945†	Winnipeg 1945	Morden 1946	Winnipeg 1947
Crystal	−.842**	−.635**	.007	−.254
Viking	−.415	−.704**	−.100	0
Royal	−.725**	−.851**	−.369	−.471*
Redwing	−.554††	−.665**	.235	−.225

* *Significant. Required for significance at 5% point, .404.*

** *Highly significant. Required for significance at 1% point, for Morden, 1945, .708; for all other station-years, .515.*

† *Disease ratings at Morden, 1945, were taken on only half the replicates.*

†† *Required for significance at 5% point, .576.*

most plots at Winnipeg in 1947 explain the low correlations for that experiment. The erratic data on oil content at Morden in 1946 can only be attributed to factors other than disease which affect oil formation in flaxseed.

The oil content of the four varieties was affected differently by the respective inoculations with pasmo, as shown by highly significant interactions between treatment and variety in all experiments except at Winnipeg, 1947. The response of each of the varieties at Morden, 1946, is illustrated in Fig. 2.

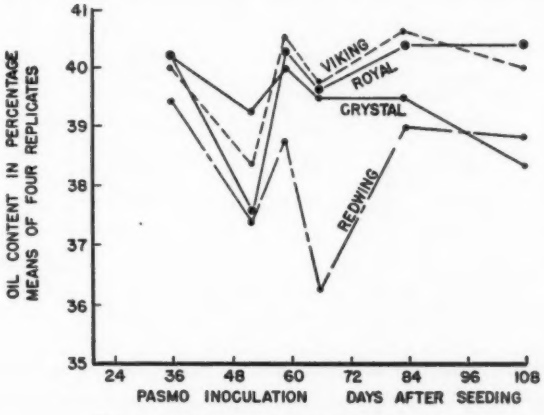

FIG. 2. Effect of date of inoculation with pasmo on oil content of four varieties of flax at Morden in 1946.

Correlation coefficients calculated for oil content and seed yield are listed in Table IV. All the values except those for Crystal at Morden, 1946, were positive, and most were significant beyond the 1% level, indicating that the factors affecting seed yield affect oil content in the same way.

TABLE IV

CORRELATION COEFFICIENTS: YIELD AND OIL CONTENT OF FLAXSEED

Variety	Morden 1945	Winnipeg 1945	Morden 1946	Winnipeg 1947
Crystal	.292	.628**	−.123	.328
Viking	.671**	.776**	.520**	.569**
Royal	.842**	.845**	.609**	.918**
Redwing	.516**	.718**	.077	.744**

** *Highly significant. Required for significance at 1% point, .515.*

The relationship between seed size and oil content is of considerable interest, as plumpness of seed is often used as a criterion of its oil content. Since yield and seed size are closely correlated (11), and yield and oil content also show a close correlation in most cases, high positive correlation between seed size and oil content might be expected. The data in Table V show the expected relationship; with the exception of Crystal at Morden in 1946, all the coefficients are positive, and most of them are very highly significant.

TABLE V

CORRELATION COEFFICIENTS: THOUSAND-KERNEL WEIGHT AND OIL CONTENT OF FLAXSEED

Variety	Morden 1945	Winnipeg 1945	Morden 1946	Winnipeg 1947
Crystal	.584**	.643**	−.253	.451*
Viking	.791**	.712**	.410*	.709**
Royal	.916**	.831**	.499*	.797**
Redwing	.667**	.672**	.207	.656**

* *Significant. Required for significance at the 5% point, .404.*
** *Highly significant. Required for significance at the 1% point, .515.*

The only published account of the effect of pasmo on the oil content of flaxseed that was seen by the authors is that of Johansen in Denmark (6). She collected and analyzed seed from slightly diseased and heavily diseased plants in a naturally infected field. The values for oil content of seed from "almost healthy" plants and diseased plants respectively were 43.42% and 43.92%. The large number of factors other than pasmo that might have affected the oil content of the seeds selected in such a manner makes it difficult or impossible to evaluate her data.

Iodine number is a measure of the drying quality of oil. High iodine number is extremely desirable in linseed oil, which is used very extensively as a drying oil in paints, linoleum, and other products. Iodine numbers were determined on oil samples from all plots. The data are presented in Table VI. Differences

TABLE VI

IODINE NUMBER OF LINSEED OIL IN WIJS UNITS. MEANS OF ALL REPLICATES

Inoculated	Days after seeding	Crystal	Viking	Royal	Redwing	Treatment mean	Minimum significant difference between means	5%	1%
Morden, 1945									
Flowering—spray	64	190.25	187.25	179.25	192.00	187.19	Treatment	1.018	1.410
Seedling—spray	39	191.75	187.50	175.00	194.00	186.81			
Seedling—straw		191.50	190.75	176.75	192.75	187.94			
Seedling—chopped straw		191.75	190.00	178.50	194.25	188.38	Variety	.673	.898
Uninoculated check	96	191.75	194.25	179.75	194.25	188.88			
Variety mean		191.22	190.18	177.35	192.80				
Winnipeg, 1945									
Flowering—spray	59	186.75	183.50	173.00	187.25	182.62	Treatment	1.352	1.873
Seedling—spray	30	188.75	187.75	172.75	189.25	184.62			
Seedling—straw		188.25	188.75	171.50	188.25	184.19			
Seedling—chopped straw		188.25	189.25	173.75	189.00	185.06	Variety	1.035	1.382
Uninoculated check	94	187.00	191.00	173.50	188.25	184.94			
Variety mean		187.55	188.39	172.93	188.18				
Morden, 1946									
Flowering—spray	51	171.25	172.50	156.25	177.75	169.44	Treatment	1.596	2.211
Seedling—spray	36	173.25	166.25	153.00	174.75	166.81			
Postflowering—spray	58	181.50	179.00	162.75	180.00	175.81			
Boll filling—spray	65	179.25	179.00	165.50	185.00	177.19			
Ripening—spray	83	183.25	184.00	162.50	181.25	177.81	Variety	1.386	1.850
Uninoculated check	108	182.25	184.50	166.50	175.25	177.13			
Variety mean		178.46	177.54	161.12	179.00				
Winnipeg, 1947									
Flowering—spray	52	182.00	181.25	159.00	184.50	176.69	Treatment	1.346	1.864
Flowering—spray	58	184.50	181.00	164.50	184.25	178.56			
Seedling—spray	40	184.25	180.50	166.50	184.25	178.62			
Flowering—spray (1/100)		184.75	185.75	168.75	187.75	181.75			
Ripening—spray	74	186.75	186.50	167.25	185.50	181.50	Variety	7.966	10.634
Uninoculated check	105	184.25	183.50	167.50	185.50	180.19			
Variety mean		184.22	183.05	165.56	185.26				

in iodine number due to treatment were highly significant in three of the four experiments and significant beyond the 5% level in the fourth, Winnipeg, 1945. Mean values for all plots inoculated at the respective dates are shown in Fig. 3.

Inoculations at flowering time reduced iodine numbers significantly below the values for uninoculated check plots in every case. Seedling inoculations at Morden in 1945 and 1946 caused even greater reductions in iodine number

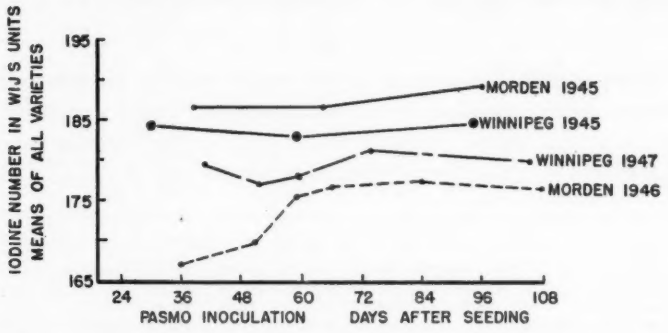

FIG. 3. Effect of date of inoculation with pasmo on iodine number of linseed oil in four station-years.

than inoculations at the flowering stage, although the difference was significant only in 1946. Seedling inoculations at Winnipeg in 1945 and 1947 caused significantly less reduction in iodine number than did inoculation at the flowering stage. Late-season inoculations in 1946 and 1947 resulted in higher iodine number values than those obtained for uninoculated check plots; although the difference was not significant in 1946, it was almost significant at the 5% point in 1947.

The correlation coefficients calculated for disease rating and iodine number are listed in Table VII. Negative correlations for all but Crystal and Royal

TABLE VII

CORRELATION COEFFICIENTS: PASMO DISEASE RATING OF FLAXSEED AND IODINE NUMBER OF LINSEED OIL

Variety	Morden 1945†	Winnipeg 1945	Morden 1946	Winnipeg 1947
Crystal	.178	−.026	−.301	−.136
Viking	−.564††	−.556**	−.595**	0
Royal	.368	−.182	−.730**	−.453*
Redwing	−.292	−.017	−.306	−.032

* *Significant. Required for significance at 5% point, .404.*
** *Highly significant. Required for significance at the 1% point, .515.*
† *Disease ratings at Morden, 1945, were taken on only half the replicates.*
†† *Required for significance at 5% point, .576.*

at Morden, 1945, and Viking at Winnipeg, 1947, indicate that pasmo apparently has an adverse effect on iodine number. The relationship is not a close one, however, as relatively few of the values are statistically significant.

Crystal, Viking, and Redwing normally have oil of high iodine number, whereas oil from Royal has a relatively low iodine number. The varieties reacted differently to the various treatments, as shown by the highly significant interactions between treatments and varieties for three of the four station years. The interaction was not significant at Winnipeg in 1947. Reactions of the four varieties to individual treatments at Morden in 1946 are illustrated in Fig. 4.

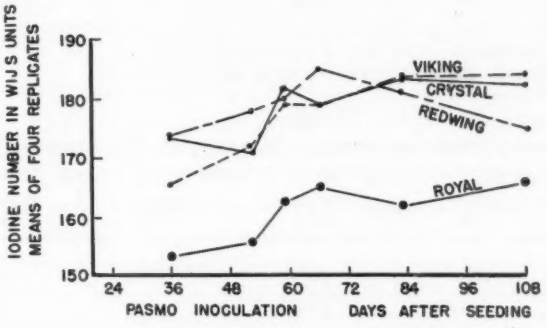

FIG. 4. Effect of date of inoculation with pasmo on iodine number of oil from four varieties of flax at Morden in 1946.

Correlation coefficients were calculated from the data for yield and iodine number, thousand-kernel weight and iodine number, and oil content and iodine number respectively. The values obtained are presented in Tables VIII, IX, and X.

TABLE VIII

CORRELATION COEFFICIENTS: YIELD OF FLAXSEED AND IODINE NUMBER OF LINSEED OIL

Variety	Morden 1945	Winnipeg 1945	Morden 1946	Winnipeg 1947
Crystal	.487*	−.121	.693**	.394
Viking	.604**	.750**	.765**	.568**
Royal	.050	.291	.711**	.814**
Redwing	.557**	.050	.313	.370

**Significant. Required for significance at the 5% point, .404.*
*** Highly significant. Required for significance at the 1% point, .515.*

TABLE IX

CORRELATION COEFFICIENTS: THOUSAND-KERNEL WEIGHT OF FLAXSEED AND IODINE NUMBER OF LINSEED OIL

Variety	Morden 1945	Winnipeg 1945	Morden 1946	Winnipeg 1947
Crystal	.297	−.007	.442*	.148
Viking	.798**	.605**	.780**	.371
Royal	−.030	.305	.595**	.578**
Redwing	.511**	.284	.350	.344

* *Significant. Required for significance at the 5% point, .404.*
** *Highly significant. Required for significance at the 1% point, .515.*

TABLE X

CORRELATION COEFFICIENTS: OIL CONTENT OF FLAXSEED AND IODINE NUMBER OF OIL

Variety	Morden 1945	Winnipeg 1945	Morden 1946	Winnipeg 1947
Crystal	.275	−.099	−.436*	.234
Viking	.890**	.696**	.355	.328
Royal	.048	.174	.375	.825**
Redwing	.554**	.392	−.342	.242

* *Significant. Required for significance at the 5% point, .404.*
** *Highly significant. Required for significance at the 1% point, .515.*

Relationships between the respective paired sets of data varied from variety to variety and from station-year to station-year. Most of the correlation coefficients were positive, and a number of them were statistically significant. It may be concluded, therefore, that the factors affecting seed yield, seed size, and oil content of flaxseed, tend to have a similar effect on the iodine number of the oil.

Discussion

The data presented in this paper indicate that severe pasmo infection results not only in lower yields of flaxseed, but also in a lower percentage of oil in the seed, and in lower iodine number of the oil. Any one of the reductions in yield or quality constitutes an important economic loss. The losses are increased because they are cumulative. Lower seed yield itself results in a lower yield of oil from a given area sown to flax. Reduction in oil content of seed harvested from diseased plants further reduces the yield of linseed oil. Reduction in drying quality of oil from pasmo-infected flax aggravates the economic loss caused by this disease.

Dillman (1) made careful determinations of the growth and oil content of flax seeds by taking seeds from plants at daily intervals from blooming to maturity. He found that seeds attained their maximum volume 12 to 14 days after the plants flowered, but decreased slightly in size during the last few days of ripening. Dry weight of the seeds increased uniformly to a maximum at 33 days, several days before the normal harvesting stage which came at 39 to 40 days after flowering. Oil formation started six days after flowering; oil percentage increased most rapidly from the seventh to the 25th day, but total oil content increased to a maximum coinciding with maximum dry weight. Painter (8) substantiated Dillman's account of oil formation, and also determined the rate of formation of the fatty acids during oil deposition, using iodine and thiocyanogen numbers as a measure of the various fatty acids present. Iodine number increased rapidly from the time oil was first formed to a maximum shortly before the oil percentage was highest, then decreased somewhat as the plants reached the usual harvesting stage. He stated that the unsaturated acids responsible for high iodine number may be formed by desaturation of other acids or, equally probably, may be formed directly and may increase in proportion during oil formation by modification in the relative rates of formation of the respective acids. Either mechanism for the increase of unsaturated acids, and consequently for higher iodine number of the oil, would be affected adversely by unfavorable environmental conditions that shortened the period of fatty acid formation. Painter, Nesbitt, and Stoa (9) found that the normal course of development is disrupted if flax is grown under adverse environmental conditions, such as a combination of drought and high temperatures. Under such conditions, both oil content and iodine number increased rapidly in the initial stages but the processes were arrested at an early stage of growth and both oil percentage and iodine number were low. Oil content might, however, reach near-optimum levels under growing conditions which inhibited the formation of high-iodine-number oils.

Dillman and Hopper (2) made an exhaustive study of the effect of climate on yield, seed size, and oil content of flaxseed, and on the iodine number of linseed oil, of four varieties of flax. They found that deficient precipitation during the growing season, and high temperatures in July, shortened the vegetative and fruiting periods. Enforced early ripening reduced yields, lowered weight per thousand kernels, and lowered oil content of the seeds and iodine number of the oil.

Greaney, Woodward, and Whiteside (4) reported that stem rust of wheat disturbed normal plant metabolism, arrested plant development, and induced early ripening. Enforced early ripening checked food movement to the seed, interfering considerably more with the deposition of organic than inorganic compounds in the kernels. As a result, much elaborated food remained in the straw and the grain was immature, small, shrivelled, and poor in protein.

Flax was affected by pasmo inoculation in much the same way as by adverse weather conditions, and in much the same way as wheat was affected by stem rust. Plant development was arrested; heavily diseased plants were shorter than healthy plants, and branched less; and ripening was accelerated. As a result, there was less material deposited in the seeds, which were immature, small, shrunken, and low in oil content.

Flor (3) found that natural infection by flax rust, like unfavorable environmental conditions, adversely affected yield, seed size, and oil content of flaxseed. Environmental conditions that reduced flax yield also reduced iodine number of the linseed oil (2). When flax yield was reduced by rust, however, the iodine number of the oil was increased. This was not the case when seed yields were reduced by pasmo infection. Sixteen correlation coefficients were calculated for the yield and iodine-number data for four flax varieties from four station-years. All but one of the coefficients were positive, and nine of them were statistically significant (eight of them highly significant). As the main factor causing variation in yields in the data reported was pasmo, the disease apparently affected fatty acid formation in much the same way as it is affected by low precipitation and high temperature.

It is of interest that Peturson, Newton, and Whiteside (10) reported variations in the effect of leaf rust of wheat on the percentage protein laid down in wheat kernels. Under certain conditions rust infection reduced the percentage of protein, and under other conditions increased it. The combination of factors that caused leaf rust to affect protein assimilation favorably in some years and unfavorably in others was not determined. It is not known whether the effect of flax rust on iodine number might vary similarly from season to season. The difference in effect of rust and of pasmo might perhaps be attributed in part to variation in climatic and other conditions between the stations and the seasons in which the respective plots were grown, and in part to the use of different varieties in the tests.

Acknowledgments

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STUDIES ON THE PRODUCTION OF VITAMIN B_{12}-ACTIVE SUBSTANCES BY MICROORGANISMS[1]

BY MARGARET O. BURTON [2] AND A. G. LOCHHEAD[3]

Abstract

Vitamin B_{12} activity, as determined by the *Lactobacillus lactis* Dorner agar plate assay, was shown by 64.6% of 537 bacteria isolated from soils, seed, manure, and poultry house litter, and by 66.6% of 676 actinomycetes from soils. For the primary isolation of active forms a simple medium without growth factors was superior to more complex media; however, the latter were more suitable for vitamin production. Further work, in which the *Lactobacillus leichmannii* assay method was used, indicated that highest yields were given by species of *Nocardia* isolated from soil, the B_{12}-active substance being chiefly associated with the cells. Vitamin B_{12} values could be increased by improving the method of extraction. Survivors of ultraviolet irradiation lethal to the great majority of treated cells were not significantly different in B_{12} producing capacity with respect to mean or dispersion when compared with nonirradiated strains.

Introduction

The production of vitamin B_{12} by microorganisms has received considerable attention since the vitamin was first obtained in crystalline form in 1948 (12, 16) and found to be a necessary growth factor for chicks (10) and pigs (7, 8). In this connection, the vitamin was recognized as the animal protein factor (APF) or one of the active constituents of the factor. In addition, vitamin B_{12} was identified as the LLD factor required for growth of *Lactobacillus lactis* Dorner (13, 15). Among the organisms used for the assay of vitamin B_{12} are *Lactobacillus lactis* Dorner, *Lactobacillus leichmannii*, a B_{12} requiring mutant of *Escherichia coli* (1), and the algal flagellate, *Euglena gracilis* (6).

Rickes *et al.* (13) reported LLD activity in culture broths of *Mycobacterium smegmatis*, *Lactobacillus arabinosus*, *Bacillus subtilis*, and several species of *Streptomyces*. Crystalline material obtained from *Streptomyces griseus* was found to be identical with vitamin B_{12}. Stokstad *et al.* (18) isolated from chicken feces an organism capable of producing APF which in concentrate was active in the treatment of pernicious anemia. The organism was subsequently identified as *Flavobacterium solare* (11). In the same laboratory (17) a B_{12}-active fraction was isolated from cultures of *Streptomyces aureofaciens*. With a strain of *Bacillus megatherium*, Lewis *et al.* (9) reported a yield as high as 800 mμgm. per ml. of culture fluid, and stated that the B_{12} activity was entirely associated with the bacterial cells.

In a survey of vitamin B_{12} production by various groups of microorganisms, Hall *et al.* (4) found various bacteria and actinomycetes, though none of the fungi or yeasts examined, capable of synthesizing vitamin B_{12}-like compounds.

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Contribution No. 319 (Journal Series) from the Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa.

[2] *Assistant Bacteriologist.*

[3] *Chief, Division of Bacteriology and Dairy Research.*

Halbrook *et al.* (3) made a study of the production of this vitamin by microorganisms isolated from poultry house litter and droppings, finding the great majority of the isolates capable of forming the vitamin in detectable amounts (more than 0.01 mμgm. per ml.). Highest production was obtained from an organism tentatively identified as a strain of *Aerobacter aerogenes*.

Tarr *et al.* (19) found that *Serratia marcescens* would grow in, and considerably enhance the natural vitamin B_{12} content of, fish solubles. This organism was one of 25 found to be capable of forming vitamin B_{12}-active substances. Hendlin and Ruger (5), in stating that large numbers of microorganisms synthesize LLD-active substances, showed that supplementation of the medium with cobalt, up to 2 p.p.m., gives rise to a significant increase in LLD activity.

Experimental

In the present investigation, a survey has been made of a large number of microorganisms for the production of vitamin B_{12}-active substances. The isolates were obtained from local soils, soils from Northern Canada, seed washings, cow manure, and poultry house litter.

Isolation of Cultures

For the isolation of the bacteria cultures, two agar media were employed, each containing 2 p.p.m. cobalt as cobalt chloride. The first medium consisted of inorganic salts and glucose and without growth factors, designed to support only those organisms capable of producing their own nutrilite requirements. The second was a complex medium containing casein digest with added growth factors without vitamin B_{12}. The various source materials were plated on either or both media, and cultured at 26° C. From suitable uncrowded plates, all colonies were picked and transferred to tubes of semisolid agar of the corresponding media. In all, 537 strains of bacteria were thus obtained. The actinomycete cultures were isolated from plates of Conn's medium made from samples of soil from various locations in Northern Canada, 676 strains being obtained which were purified by replating.

Testing for B_{12} Production.

The bacteria were tested for B_{12} production in liquid media by transferring from the semisolid agar stock culture to broth tubes corresponding to the original isolation medium. After growth was well established, transfers were made by addition of two drops to 20 ml. of the test medium in 125 ml. Erlenmeyer flasks. It most cases two or three test media of different composition were employed (all with 2 p.p.m. cobalt), as follows:

(i) — inorganic salts, glucose
(ii) — (i) plus organic nitrogen (vitamin-free casein digest)
(iii) — (ii) plus growth factors, excluding B_{12}

After 8 to 10 days' incubation at 26° C. the culture broths were adjusted to approximately pH 6.8, sterilized at 15 lb. pressure for 15 min., and examined by the cup assay method of Foster *et al.* (2) employing the test organism

Lactobacillus lactis Dorner (ATCC No. 10,697) and vitamin B_{12} as standard. The actinomycetes were tested qualitatively for LLD activity by spotting spore suspensions on plates of Conn's agar (with added cobalt) and, after four to five days at 26° C., flooding with Foster's agar plate assay medium seeded with *L. lactis* and incubating at 37° C.

Results

As shown in Table I, vitamin B_{12} activity in amounts detectable by the *L. lactis* plate assay method, was produced by 64.4% of the bacteria and by 66.6% of the actinomycetes. Organisms from all the sources studied showed

TABLE I

PRODUCTION OF VITAMIN B_{12}-ACTIVE SUBSTANCES BY MICROORGANISMS FROM VARIOUS SOURCES

Source	No. of cultures isolated	No. of B_{12} producers	% B_{12} producers
Bacteria			
Local soil	126	79	62.7
Northern soils	127	107	84.3
Seed washings	48	23	47.9
Cow manure	92	58	63.0
Poultry litter	144	80	55.6
Total	537	347	64.6
Actinomycetes			
Northern soils	676	450	66.6

a high proportion of active forms. Robbins *et al.* (14) have stated that as many as 50% of the organisms developing on plates seeded with soil were found to produce vitamin B_{12}, as determined by the *Euglena* assay procedure.

TABLE II

INFLUENCE OF ISOLATION MEDIUM ON THE PERCENTAGE OF VITAMIN B_{12}-PRODUCING BACTERIA FROM VARIOUS SOURCES

Source	Isolation medium					
	Simple (minus growth factors)			Complex (plus growth factors)		
	No. of isolates	No. of B_{12} producers	% B_{12} producers	No. of isolates	No. of B_{12} producers	% B_{12} producers
Local soil	74	52	70.3	52	27	51.9
Northern soils	127	107	84.3	—	—	—
Seed washings	—	—	—	48	23	47.9
Cow manure	67	48	71.6	25	10	40.0
Poultry litter	82	51	62.2	62	29	46.8

The advantage of using a simple medium for the primary isolation of bacteria able to produce the vitamin is brought out in Table II. This medium, without growth factors, shows a selectivity by permitting the development of a higher percentage of organisms capable of B_{12} synthesis than the more complex medium. However, though the simple medium is of advantage for the purpose of isolation, it is less suited to production of the vitamin than richer media. This is shown in Table III, in which the amounts of vitamin B_{12} produced in

TABLE III

VITAMIN B_{12} PRODUCTION IN DIFFERENT MEDIA BY BACTERIA FROM VARIOUS SOURCES

Source		Medium for testing B_{12} production		
		Synthetic (inorg. N)	Casein hyd. (amino acids)	Casein hydrol. gr. factors
Soil	No. of cultures tested	118	119	117
	B_{12} positive	72 (61.0%)	102 (85.7%)	73 (62.4%)
	Zone*, under 10 mm.	46 (63.8%)	9 (8.8%)	6 (8.2%)
	" 10–15 mm.	19 (26.4%)	17 (16.7%)	31 (42.5%)
	" over 15 mm.	7 (9.8%)	76 (74.5%)	36 (49.3%)
Seeds	No. of cultures tested	41		34
	B_{12} positive	11 (26.8%)		16 (47.2%)
	Zone, under 10 mm.	4 (36.4%)		4 (25.0%)
	" 10–15 mm.	5 (45.4%)		10 (62.5%)
	" over 15 mm.	2 (18.2%)		2 (12.5%)
Manure	No. of cultures tested	13	70	74
	B_{12} positive	4 (30.8%)	39 (55.8%)	46 (62.1%)
	Zone, under 10 mm.	2 (50.0%)	7 (17.9%)	14 (30.5%)
	" 10–15 mm.	2 (50.0%)	22 (56.4%)	18 (39.2%)
	" over 15 mm.	0 (0.0%)	10 (25.7%)	14 (30.5%)

* *Diameter of zone of growth, L. lactis cup assay method.*

test media of varying complexity by bacteria from three sources are compared in terms of zone sizes by the *L. lactis* plate agar assay procedure. As indicated by the zone diameter, higher average amounts of vitamin were produced in the more complex media than in the simple medium where growth was usually less abundant.

Effect of Cultural Conditions and Assay Procedures

Sixty-three bacterial isolates showing the highest yields of vitamin B_{12} were selected for studies with various media, aeration conditions, and temperatures. In a basal glucose–salt solution, different nitrogen and vitamin sources were tested, including casamino acids (Difco), soybean meal, peptone, meat extract, sodium nitrate, and yeast extract. The majority of the organisms gave the best response on a 'casamino acid' medium which contained per liter, dipotassium phosphate, 1.0 gm.; potassium nitrate, 0.5 gm.; $MgSO_4 \cdot 7H_2O$, 0.2 gm.;

calcium carbonate, 0.1 gm.; sodium chloride, 0.1 gm.; $FeCl_3 . 6H_2O$, 0.01 gm.; $CoCl_2 . 6H_2O$, 0.008 gm. After this salt mixture was heated and filtered, the following were added: casamino acids (vitamin free), 5.0 gm.; glucose, 10.0 gm.; and yeast extract, 1.0 gm. The pH was adjusted to 7.2. The medium was dispensed in 80 ml. quantities in 300 ml. Erlenmeyer flasks, inoculated, and incubated in shake culture at 26° C. for six to eight days. The inoculum was prepared by washing the growth from a 48-hr. 'casamino agar' slope with 2 ml. of sterile saline and adding 0.5 ml. of this suspension to each flask of medium.

With the cup plate assay of culture fluids with *L. lactis*, two types of zones were obtained. One was a dense, clearly defined zone typical of that given by crystalline vitamin B_{12} in solution; the other was less dense, with ill-defined border. In order to check the results obtained with the plate assay and to measure more accurately the B_{12} effect given by the atypical zones, a titration assay was used employing as test organism *Lactobacillus leichmannii* (ATCC 4797) with Difco vitamin B_{12} assay medium. Cultures showing dense zones of 20–26 mm., corresponding to 50 to over 200 mμgm. B_{12} per ml., gave invariably lower values by the titration assay. Three *Pseudomonas* cultures, giving large, fuzzy zones up to 40 mm. in diameter (over 500 mμgm. B_{12} per ml.), showed only 30 to 60 mμgm. B_{12} per ml. by the titration method. This discrepancy between the assays was not due entirely to different responses by the two test organisms, for culture fluids retested by a *L. leichmannii* cup plate assay gave B_{12} values paralleling those obtained with the *L. lactis* agar method, considerably higher than those indicated by the titration assay with the same strain of *L. leichmannii*. Although the plate assay is valuable for screening purposes, the titration assay is much more sensitive and is recommended for more accurate determinations. This assay was adopted for the remainder of the work.

Three bacterial isolates which gave the highest yields by the titration assay were selected for further study. Two of these cultures were isolated from local soil and one from a soil sample from Northern Canada and upon examination proved to be species of *Nocardia*. The yields obtained from these organisms varied from 150–200 mμgm. of vitamin B_{12} per ml. of culture fluid.

Pretreatment of Cultures for Assay

In cultures of *Nocardia* most of the B_{12} activity was found to be associated with the cells. Assays of centrifuged culture fluids and cells respectively showed approximately 92% of the total activity to be in the latter. It was found that the assay values could be raised by improving the method of extraction of the vitamin from the cells. The technique used in preparing samples for titration assay had been to adjust the pH of culture fluids to approximately pH 6.8, dilute, and add appropriate amounts to the assay tubes prior to sterilization. However, it was found that autoclaving the acidified culture fluids from 5 to 20 min. before assaying increased the yield.

In addition, the pH to which the culture material was adjusted prior to autoclaving was found to influence the vitamin B_{12} yield. Furthermore, different values were obtained depending on whether the material to be assayed was sterilized with the assay medium or added aseptically after sterilization. This was shown when identical culture broths were adjusted over a pH range of 2.0 to 7.0, autoclaved 12 min., diluted, and added to the assay tubes before and after sterilization, respectively. As shown in Fig. 1, higher yields

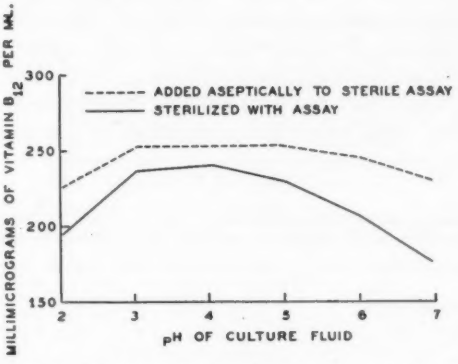

FIG. 1. Effect of pH of culture broths on yield of vitamin B_{12}.

of the vitamin were obtained at all pH levels when the material was added to the assay aseptically rather than when it was sterilized with the assay. The pH for optimum yield of the vitamin lay between 3.0 and 5.0 in both cases. A parallel experiment with pure vitamin B_{12} in 'casamino acid' broth showed no difference in B_{12} content when the material was added to the assay both before and after sterilization. There was a slight depression in yield at pH 2.0 and pH 7.0 in both cases, with no optimum pH apparent.

The technique recommended for the vitamin B_{12} assay of *Nocardia* culture broth is to adjust the pH to 4.0, heat for 5 to 10 min. at 15 lb. pressure, and dilute the material to the assay range of 0.01 to 0.10 mμgm. of vitamin B_{12} per tube. For optimum yield of the vitamin, the diluted material is added aseptically to the sterile assay medium. By using this technique, up to 320 mμgm. of vitamin B_{12} per ml. of culture fluid have been obtained with a strain of *Nocardia* (No. SS112).

Irradiation of Cultures

A culture of SS112 was subjected to ultraviolet irradiation in an attempt to obtain variants which could produce vitamin B_{12} in quantities greater than the yield given by the parent strain. Dilutions of 1 : 10,000 and 1 : 100,000 of a broth culture of SS112 were spread in 0.1 ml. quantities on 'casamino acid' agar plates and allowed to dry. Series of plates were exposed to ultraviolet

irradiation for 0, 0.5, 1.0, 2.0, 3.0, and 5.0 min. respectively and incubated at 26° C. for four days. Ultraviolet irradiation was provided by a 15-watt G.E. germicidal lamp (2,537 Å ultraviolet) at a distance of 22 in. Numbers of colonies decreased markedly with increased time of irradiation, from "uncountable" in control plates to 100% kill in plates exposed for five minutes. One hundred colonies were selected at random from the plates exposed for 3.0, 2.0, 1.0, and 0.5 min. These, when cultured and tested for B_{12} production gave values which varied from 45–360 mμgm. per ml. To determine the effect of irradiation in causing this range of values, 100 nonirradiated colonies were tested and showed yields varying from 80–300 mμgm. per ml. of culture fluid. These values, expressed as percentage of the nontreated control, ranged from 54.5 to 154.7 for nonirradiated and from 29.4 to 170.6 for irradiated cultures. The distribution of values is recorded in Table IV.

TABLE IV

VITAMIN B_{12} PRODUCTION BY IRRADIATED AND NONIRRADIATED STRAINS OF *Nocardia* SP. EXPRESSED AS PERCENTAGE OF CONTROL CULTURE

B_{12} values as % of control	Number of strains	
	Nonirradiated	Irradiated
0 – 30	0	1
30.1 – 50	0	1
50.1 – 70	5	5
70.1 – 90	15	24
90.1 – 110	40	28
110.1 – 130	30	27
130.1 – 150	8	12
150.1 – 170	2	1
170.1 – 190	0	1
Total	100	100

Although there appears to be a greater spread of values with the irradiated cultures, statistical analysis of the data indicated that the irradiation treatment was without significant effect on the mean or dispersion with respect to yield. Though the highest yield was obtained from an irradiated strain, subsequent variability in vitamin producing capacity does not support its being considered a mutant.

Acknowledgments

The authors are indebted to Mr. G. B. Landerkin for providing the organisms isolated from Northern Canadian soils and to Dr. R. H. Wallace for the bacteria from seeds. Thanks are also due to Mr. T. M. Heringer for technical assistance and to Dr. G. B. Oakland and Miss Constance E. Cox, for the statistical analysis of the data from the irradiation tests.

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STUDIES ON THE LEGUME ROOT NODULE BACTERIA

I. DETECTION OF EFFECTIVE AND INEFFECTIVE STRAINS[1] *

By D. C. Jordan[2] and E. H. Garrard[3]

Abstract

The Virtanen technique was used to detect effective and ineffective rhizobia. The rhizobia studied varied widely in their action towards different varieties of the same host species. Maximum leghemoglobin concentrations occurred just prior to blossoming in field legumes, but much earlier in greenhouse legumes cultivated under conditions of this experiment. The nodules of the ineffective strains of legume bacteria differed from those produced by effective strains in color, size, distribution, rate of destruction of the bacteroid area, and presence or absence of rodlike bacterial forms. Experimental evidence is presented to show that a supposedly parasitic strain of alfalfa organism can exist.

Introduction

It is known that different strains of root nodule bacteria vary widely in their ability to benefit the host plant. Bacteria fixing an inadequate supply of atmospheric nitrogen for optimum plant development usually are termed ineffective. Reports indicate that ineffective rhizobia have occurred among several legumes and perhaps are more widespread than was formerly believed. Helz *et al.* (7) reported the presence of organisms that produced nodules but which did not benefit the host, while Keeney (9) found ineffective strains among clovers. Leonard (10) attributed failure of Austrian winter peas to ineffective root nodule bacteria, and Leonard and Dodson (12) produced a good crop of Austrian winter peas inoculated with efficient bacteria and grown in a soil known to contain ineffective nodule bacteria. Thornton (16) reported the presence of ineffective strains on clovers and indicated that, in addition to being useless to the host, they may prevent the entrance of useful strains.

A review of the literature reveals only vague references to ineffective strains of root nodule bacteria being parasitic in nature. As early as 1890, Frank (6) and later, Schneider (14) claimed the bean bacteria to be purely parasitic. Parasitism has been reported by Allison (1) and Bond (4) as the result of decreased carbohydrate supply. Other workers, including Baldwin and Fred (2), Hooper (8), Bjälfve (3), and Nutman (13) have reported cases of parasitism but not under controlled conditions. Thornton (17) found that alfalfa and clover bacteria may become parasitic under certain host conditions. On the contrary, Wilson (18) reports that rhizobia are not parasitic in nature.

The main obstacle in correctly assessing the effectiveness of root nodule bacteria has been, that up to the present, there has been no yardstick available

[1] *Manuscript received March 29, 1951.*
Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ont.

[2] *Assistant in Research.*
[3] *Professor and Head of Department.*

* *Part of the programme of the Legume Research Committee in Ontario.*

to measure degrees of ineffectiveness or parasitism. As workers have shown that an abundance of nodules on the root system of the host is not necessarily a criterion of successful nitrogen fixation it would appear that in order to truly evaluate the effectiveness of root nodule bacteria, it is necessary to proceed further than the commonly accepted method of comparing the macroscopic appearance of inoculated and uninoculated plants grown in artificial media, soil, or sand under similar conditions. The discovery by Virtanen (19, 20) that the efficiency of nitrogen fixation can be detected by chemical means prompted a series of investigations to determine the value of the technique in establishing well defined limits between effective, ineffective, and perhaps parasitic strains of rhizobia, to study the factors responsible for strains becoming ineffective, and to attempt to determine the prevalence and importance of such strains of root nodule bacteria.

Experimental

Field Studies

The Virtanen Technique

This procedure is based upon the detection of the presence or comparative absence of a hemoglobinlike pigment, termed leghemoglobin by Virtanen, found within the nodules of leguminous plants. This substance is formed by the combined activities of both plant and bacteria neither being able to produce it alone. After the cessation of vegetative growth, this reddish pigment is converted into a green material, a process which has been found to be analogous to the conversion of animal hemoglobin into bile pigments. Indeed, a well characterized bile pigment, biliverdin, has been prepared from the green pigment (21).

Virtanen (19, 20) found a direct relationship between the concentration of leghemoglobin and the amount of molecular nitrogen fixed during the symbiotic process. This correlation has been recently corroborated by Smith (15). It therefore appeared that the efficiency of nitrogen fixation in nodules could be estimated by the use of leghemoglobin determinations.

The technique used was a slight modification of that developed by Virtanen. It took the form of a simplified chemical procedure, standardized to ensure comparable results.

Nodules were excised from legume roots, washed, air dried for 15 min. on blotting paper, and accurately weighed. An attempt was made to keep the total weight of the nodules above 0.5 gm. The nodules were then crushed in a mortar with an excess of sodium hyposulphite and extracted twice with cold pyridine, using centrifugation. The total amount of pyridine used did not exceed 9.5 ml. The extracts were added together and made up to a total volume of 10 ml. with pyridine, using a volumetric flask. Mixing was accomplished by inverting the flask four times. The fluid was then centrifuged to throw down any contaminating particles and the clear supernatant collected.

The per cent transmittance of this supernatant was immediately determined with a Coleman spectrophotometer, using a 6-310 cuvette and a wave length of 550 mμ. The number of gamma of leghemoglobin, determined as pyridine hemochromogen, per gram of nodular tissue was then calculated from a calibration graph.

The calibration graph had been plotted from values obtained by treating chicken blood with hyposulphite and pyridine. The hemoglobin content of the blood was previously determined with the spectrophotometer using a standard procedure. A spectroscopic and spectrophotometrical analysis of this chicken blood extract revealed absorption maxima, at 555 and 525 mμ (Fig. 1).

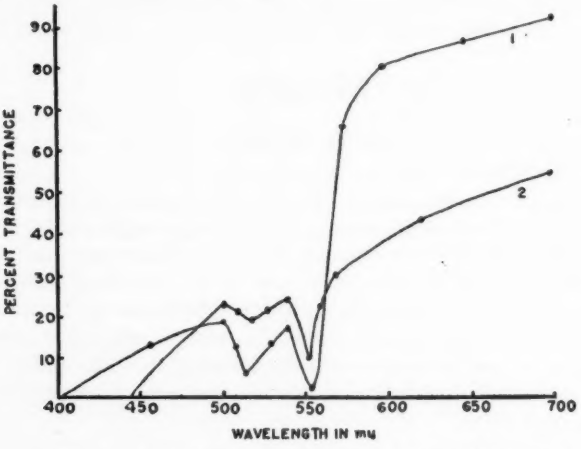

FIG. 1. Spectral transmittance graphs of (1) chicken blood extract and (2) nodule extract, showing their similarity to each other.

In previous work, however, a 550 wave length had been used and it was thought advisable to adhere to this value so that results would be comparable.

Methods

It had been found in previous studies* that a curve of leghemoglobin fluctuation existed over the growing season of the host plant. The expression of this curve was macroscopically visible in the transition of white nodules, such as are observed on very young legumes, into pink and red nodules. Studies on soybeans had revealed the following:—

1. A varietal difference existed in the host plants, both in regard to the maximum concentrations of leghemoglobin produced and in the rate at which the peak productions were reached (Fig. 2).

* *Annual report of the Ontario Legume Research Committee, 1949.*

2. The peak concentrations of pigment were attained just prior to blossoming, after which point the nodules became green. This confirmed Virtanen's findings.
3. When the nodules became green all leghemoglobin determinations had to be discontinued because of interference effects.

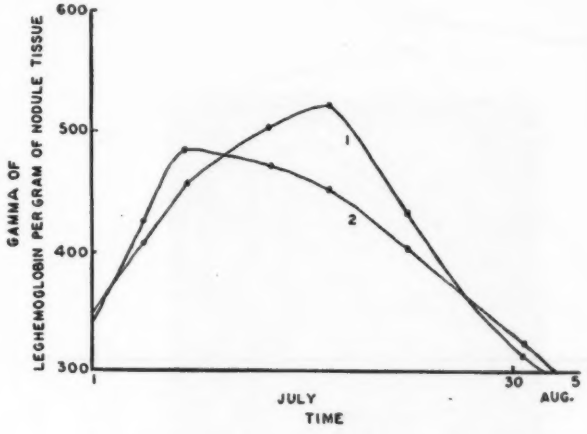

FIG. 2. Graphs showing the varietal hemoglobin variations in field plot soybeans, 1949 data. (1) Variety Goldsoy. (2) Variety Pagoda.

These points were all taken into consideration during the present work when leghemoglobin analyses were performed on O.A.C. 1 alfalfa, Alon alsike, and vetch. These legumes were grown in field plots on the College experimental farm and were not artificially inoculated. Nodulation, then, was the result of infection by "wild" rhizobia.

This field work was carried out to determine whether maximum leghemoglobin concentration was reached just prior to blossoming as with soybeans, and whether the "wild" rhizobia present in the nodules were efficient in nitrogen fixation.

Legume plants were selected from their respective plots at regular intervals throughout the growing season. The plants were selected so that they were representative of the entire plot. The root nodules were excised and the pigment concentrations found using the Virtanen technique.

Results

All the species of legumes examined showed excellent growth and were of a healthy dark green color.

Fig. 3 shows the leghemoglobin variations for the three legume species examined. The maximum concentrations were in excess of 300 gamma per gram of nodule tissue and in all cases these peaks were reached just before blossoming time.

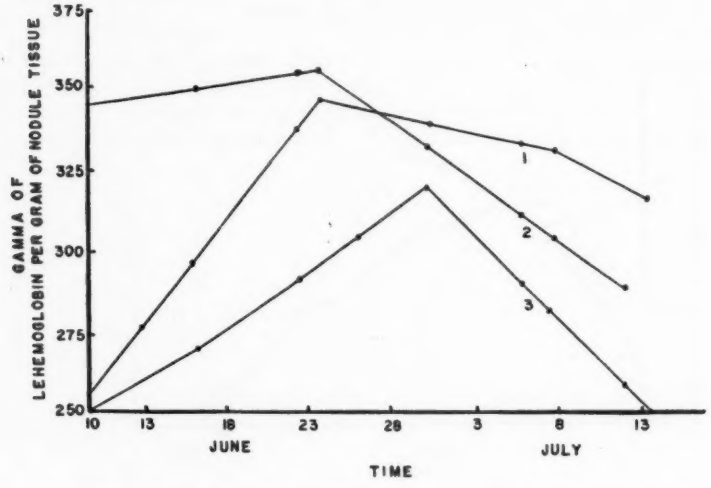

FIG. 3. Graphs showing the peak concentrations of leghemoglobin found in field plot tests. (1) O.A.C. 1 alfalfa. (2) Alon alsike. (3) Vetch (*Vicia villosa*).

GREENHOUSE STUDIES

The tremendous interplay of biological and chemical forces within the soil complicates the picture of the natural processes leading to nodulation and eventual nitrogen fixation. Certain soil constituents may, by their presence or absence, exert definite influences upon the symbiotic processes. An excess of combined nitrogen or a deficiency of such elements as boron and molybdenum would provide an environment deleterious to optimum nodulation and fixation. Various soil organisms, through an antagonistic or beneficial influence, could also modify or distort the normal sequence of events. A combination of these and many other factors would actually represent what might be termed "natural" conditions. Since there is no such thing as a "natural" or "normal" soil it is necessary to eliminate these external conditions as much as possible, at the same time providing an environment beneficial to plant growth. For this reason greenhouse experimentation was carried out.

This phase of the work was arranged so as to meet certain obligations which were necessary for a true evaluation of experimental results. It was performed under bacteriologically controlled conditions, in surroundings free of combined

PLATE I

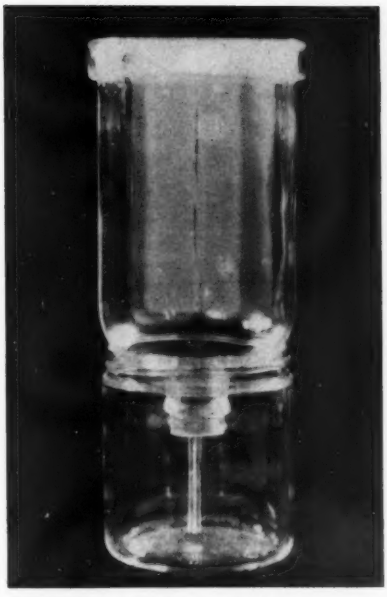

FIG. 4. Sterile glass assembly for plant growth under bacteriologically controlled conditions.

nitrogen, and with the utilization of a sufficient number of test plants to randomize errors that may have been introduced by genetical variations within the host plants.

Methods

A. Preparation of Assemblies

Table I shows the legumes tested, the inocula used, and the number of plant growth assemblies involved in each case.

TABLE I

TABLE SHOWING LEGUME VARIETIES TESTED, INOCULA, AND NUMBER OF ASSEMBLIES USED IN EACH CASE

Host plant	Inocula	No. of assemblies used
Grimm alfalfa	R_{20}, R_{21}, R_{23}	168
O.A.C. 1 alfalfa	R_{20}, R_{21}, R_{23}	168
Vicia villosa	R_2	56

All the legumes were grown in sterile sand and were kept under completely aseptic conditions until they reached a height of 4-5 in. The glass apparatus previously described by Leonard (11) was used for this purpose (Fig. 4). It consisted of two glass chambers, connected by a glass capillary tube. The upper chamber contained sand and the lower one was filled with nutrient solution. In preparing these glass assemblies for use, coarse sand was thoroughly washed with distilled water, oven dried, sieved through holes 2 mm. in diameter, and used to fill the upper chambers. The lower chambers were two-thirds filled with nutrient solution which was drawn up into the sand by the application of suction. Petri dish tops were then placed over the upper openings and the entire assemblies sterilized for 30 min. at 15 lb. pressure. This time and pressure were found to effect complete sterilization, as shown by the absence of growth when sand from the interior regions of the assemblies was plated out on nutrient agar. The nutrient solution used was a nitrogen-free modification of that used by Davis and Hoagland (5).

B. Planting, Inoculation, and Care of Assemblies

The seeds used were surface sterilized by soaking them in concentrated sulphuric acid for 20 min., followed by three washes in sterile distilled water. The treated seeds gave no evidence of bacterial or mold contamination when placed in nutrient broth and incubated for seven days.

The seeds were aseptically planted by thrusting them into the sand with sterile forceps. Eight seeds were planted in each assembly.

The seeded assemblies were kept in the dark until germination was complete. When the young plants had reached a height of approximately 1 in. they were inoculated with a 0.85% saline suspension of the specific *Rhizobium*, prepared from a 72 hr. agar slant culture and 1 liter of saline. Ten milliliters of this suspension were added to each assembly, placing the fluid around the base of each plant. After inoculation the assemblies were removed to the greenhouse and randomized on the benches. No attempt was made to completely plan the experiment for statistical analysis because of the limited bench space available and the lack of the very large number of glass assemblies that would have to be required.

Dust was reduced to a minimum in the greenhouse by repeatedly washing down the benches and floor with water and by occasionally pouring a commercial disinfecting solution over the cement walks.

When the glass covers were eventually removed, the sand was washed with sterile glass distilled water, and the plants aseptically thinned where required. Subsequent feeding with nutrient solution was accomplished by surface additions, the nutrient solution being added once every four waterings. Care was taken not to splash solution or sand from one assembly into another.

The efficiency of the greenhouse procedures in preventing contamination by foreign rhizobia was shown by the fact that only very rarely did the control plants show any sign of nodulation.

C. Examination Procedures

Periodically the plants were examined and when nodules had developed assemblies were selected by lot, and leghemoglobin determinations carried out on the nodules. Usually from four to six assemblies, involving 16 to 24 plants, were initially used in order to supply a sufficient weight of nodules. These determinations were repeated at definite intervals and concluded when the nodules began to turn green.

The dry weights of inoculated and control plants were formed by placing the green "above ground" parts in an oven held at 100° C. until the weights remained relatively constant.

A microscopic and macroscopic examination of the nodules from various plants was carried out in certain cases and involved both section and smear preparations. Nodule sections were made with the paraffin technique and stained with hematoxylin and orange G. Smear preparations were made by crushing nodules between two glass slides and staining with 0.1% aqueous crystal violet.

Results

The inoculated greenhouse vetch plants were yellow, stunted, and resembled the uninoculated controls in general appearance. A typical curve of

leghemoglobin concentration was found (Fig. 5) but the peak concentration was only 54.86 gamma. The dry weights of the tops of the inoculated plants were, however, considerably higher than those of the controls (Fig. 6).

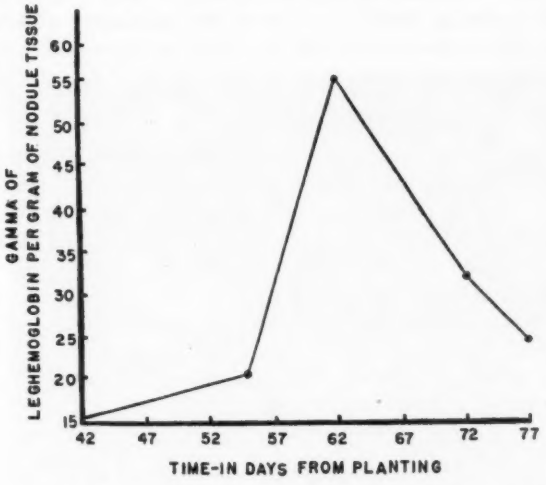

FIG. 5. Graph resulting from greenhouse vetch determinations showing the peak concentration of leghemoglobin produced within the nodules.

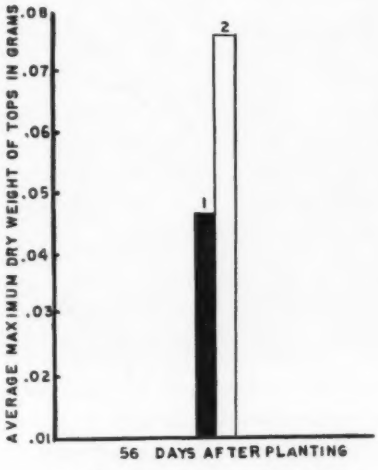

FIG. 6. Bar chart showing the comparison between the average maximum dry weights of (1) control vetch and (2) inoculated vetch.

The alfalfa plants inoculated with the R_{21} and R_{23} strains of rhizobia were healthy and vigorous (Fig. 7, 2 and C.) Figs. 9 and 10 show the average maximum pigment concentrations and the highest average dry weights of the greenhouse alfalfa. The nodule bacteria varied in their ability to benefit the two alfalfa varieties, similar to the results previously observed with soybeans. The R_{23} bacteria produced the greatest gains with the Grimm alfalfa while the R_{21} bacteria were more efficient on the O.A.C. 1 variety.

Parasitism

Without exception, all the R_{20} inoculated alfalfa plants were yellow and stunted (Fig. 7, A) and all those remaining at the conclusion of the experiment died. When some of these plants were fed with a solution of combined nitrogen (potassium nitrate) they became dark green and rapidly increased their growth, thus indicating a state of acute nitrogen starvation.

The leghemoglobin values for the R_{20} nodules are not shown because the transmittance values of the nodular extracts approached 100% T. A considerable amount of error, therefore, would be inherent in any calculations. It was, however, plainly visible, that the almost completely white R_{20} nodules contained less pigment than the very slightly pink vetch nodules.

Figs. 9 and 10 show that with both alfalfa varieties the dry weights of the R_{20} inoculated plants fell below those of the uninoculated control plants. It is mainly because of this that the R_{20} strain of nodule bacteria has been designated "parasitic" rather than simply "ineffective" which would have been the case if the inoculated plants had not suffered as a direct result of the symbiotic relationship.

The R_{20} plants possessed numerous, small, white, nodules which were uniformly dispersed over the root system (Fig. 8, B). The nodules produced by the R_{21} and R_{23} bacteria were comparatively large, pink in color, and clustered about the upper portion of the root system (Fig. 8, A).

A microscopic study made 28 days after planting revealed that the R_{20} nodules exhibited an early necrosis of the bacteroid areas similar to that observed in very old nodules prior to their being sloughed off from the root system. No such tissue destruction was evident in the R_{21} and R_{23} nodules until quite late in the growing season, as would be expected. The vetch nodules seemed to appear somewhat similar to the R_{20} nodules, but at the time of examination the necrosis was not at such an advanced state. Nodule smears showed only rod-shaped bacterial forms in the R_{20} nodules, while bacteroid forms predominated in the red nodules of the R_{21} and R_{23} nodule bacteria. The green nodules of the latter bacteria contained mainly rods with comparatively few bacteroids being present.

In order to eliminate the possibility of a bacteriophage specific against the R_{20} strain of *Rhizobium* being present, an endeavor was made to isolate such an agent from the culture sand. No evidence of any lytic agent was found, however, and the 'phage' hypothesis was discarded.

PLATE II

FIG. 7. Grimm alfalfa, eight weeks—(A) inoculated with R_{20} *Rhizobium*; (2) inoculated with R_{21} *Rhizobium*; (C) inoculated with R_{23} *Rhizobium*; (D) control—not inoculated.

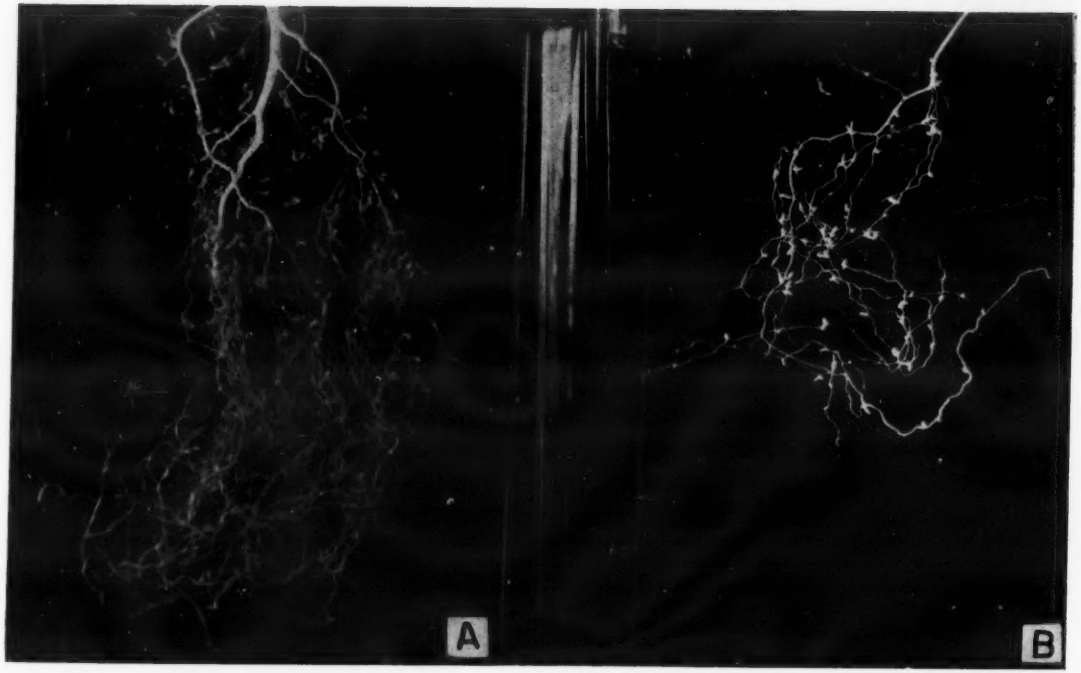

FIG. 8. Alfalfa roots, eight weeks—(A) from a plant inoculated with R_{23} *Rhizobium*; (B) from a plant inoculated with R_{39} *Rhizobium*.

Rhizobia were isolated from nodules at certain intervals and the isolates identified with their respective parent cultures by a combination of cultural and fermentation tests, and serological reactions involving the use of immune rabbit sera.

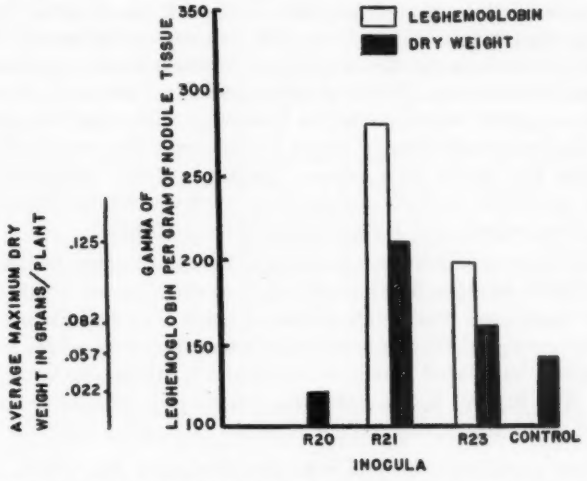

FIG. 9. Bar chart of O.A.C. 1 alfalfa showing the maximum concentration of leghemoglobin and the maximum dry weights resulting from the use of three different rhizobia strains.

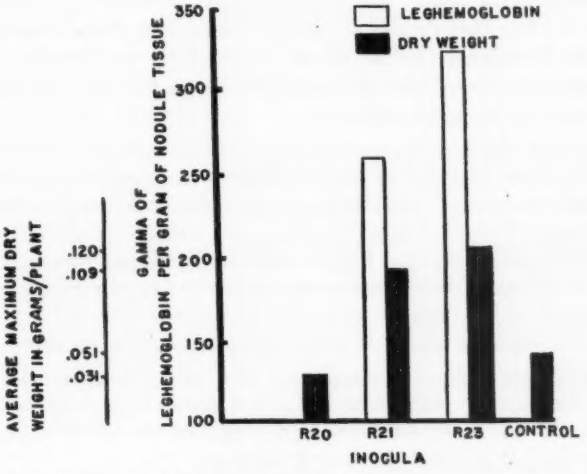

FIG. 10. Bar chart of Grimm alfalfa showing the maximum concentration of leghemoglobin and the maximum dry weights resulting from the use of three different rhizobia strains.

Discussion

These studies appear to indicate that the Virtanen technique for the determination of nodule hemoprotein may be of considerable importance in the detection of effective and ineffective strains of nodule bacteria. Since the highest concentrations of leghemoglobin in field legumes occur just prior to blossoming, determinations made at this critical period would enable an investigator to make accurate comparisons between various legumes. In the greenhouse, under the conditions of this experiment, the peak concentrations of pigment occurred some time before flowering. This may have been due to a retardation of growth brought about by the somewhat restricted conditions under which the plants were grown. Because of this, greenhouse legumes should be examined periodically so that a curve can be drawn and the maximum concentration of leghemoglobin thus detected.

The field work revealed that the "wild" types of nodule bacteria studied were efficient in nitrogen fixation according to the Virtanen method. Under controlled conditions the vetch organism used was definitely ineffective. Growth was poor and the leghemoglobin concentration was low, but the dry weights of the inoculated plants were superior to those of the uninoculated controls. The R_{21} and R_{23} rhizobia were effective, producing healthy plants with a high concentration of nodular pigment and having high dry weights.

Under the experimental conditions described, the R_{20} alfalfa *Rhizobium* appeared to be truly parasitic. The pigment values were extremely low and may not have been indicative of leghemoglobin at all, but merely representative of small amounts of other hemoproteins such as catalase or components of the cytochrome system. The state of parasitism was more clearly demonstrated by the fact that the dry weights of the control plants were superior to those of the inoculated plants. If the R_{20} bacteria were simply ineffective then it would be expected that the inoculated plants would not be significantly different from the uninoculated ones.

The fact that the parasitic nodules differed from effective nodules in size, distribution, color, and rate of decomposition is in complete agreement with the available literature. It is believed that the abortive disintegration of essential tissue is a manifestation of a decreased carbohydrate supply produced by subnormal plant growth. Under otherwise optimum growing conditions a lack of sufficient carbohydrate could be the result of nitrogen starvation brought about by infection by ineffective or parasitic rhizobia. Under such conditions the bacteria appear to attack the plant cells as an energy source.

Changes in carbohydrate synthesis may also induce morphological changes in the nodule bacteria, but since the actual status of the various forms of rhizobia is not well understood, the significance of the bacterial types found in the effective and parasitic nodules is unknown.

It is not known whether the parasitism of the R_{20} bacteria had been present upon initial isolation or had developed during laboratory cultivation. If the

period of artificial cultivation had induced such a drastic change in effectivity then it was limited to the R_{20} strain only, because neither of the other two strains developed similar tendencies.

Greenhouse conditions, being artificial in nature, may not parallel field conditions. The use of field trials to substantiate greenhouse results is, however, contraindicated by the large number of soil factors tending to influence fixation. Nevertheless it seems plausible that any strain of nodule bacteria acting as a parasite under controlled conditions is potentially capable of similar action in the field.

The exact mechanism by which parasitic and ineffective rhizobia arise is largely speculative. They may occur in soil because of normal mutation and subsequent environmental selection or through the activity of mutagenic agents of undetermined origin. Such mutations might not only result in a loss of essential enzyme systems connected with the fixation process, but could also produce additional changes which in some way would result in detrimental influences upon the host. These latter changes could conceivably be related to the elaboration of a phytotoxic substance which the plant could absorb via its vascular system.

A wide range in the ability of rhizobia to benefit their hosts appears to exist. This range includes gradations involving effective, ineffective, and parasitic bacteria with no clear-cut differentiation among them. Bacteria and host must be studied together in this respect since it is only through an intimate relationship with each other that the various degrees of effectiveness become evident.

Acknowledgments

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OBSERVATIONS ON QUATERNARY AMMONIUM DISINFECTANTS[1]

By C. E. Chaplin

Abstract

Bacterial death rate studies were carried out with several quaternary ammonium compounds. The survivor curves were found to be sigmoid. This shape was shown to be due to the difference in resistance to the QAC among the individual cells. An exponential rate was found when a more resistant strain was selected from the survivors of disinfection. Sporocidal action was not found. The reaction between substrate protein and the QAC removes the disinfectant from solution and occurs in the presence of a wide QAC–protein ratio, but the bactericidal action is dependent upon the concentration of the disinfectant. *Serratia marcescens* was adapted to grow in 200 times the originally lethal concentration of disinfectant, *Escherichia coli* in 43 times, but no increase in concentration was tolerated by *Micrococcus pyogenes* var. *aureus*.

Introduction

Quaternary ammonium compounds (QAC) have presented numerous difficulties to investigators since their rise to prominence among disinfectants. It is fairly well established now that the phenol coefficient method for evaluating germicidal potency, because of the prevalence of "skips" and "wild pluses", cannot be intelligibly applied to these substances (1, 2, 8).

During a search for a more reliable modification of the phenol coefficient test or a replacement for it, it was found necessary to investigate the behavior of the QAC from a number of viewpoints, a few of which are discussed herewith.

Death Rates

Recently (11, 12) it has been claimed that the death rates of bacteria under the influence of QAC show a curve asymptotic to the time axis. Since high concentrations of interfering substances were used during those studies the death rates reported cannot be regarded as a true representation of the action of QAC on bacterial cells. It was, therefore, decided to use aqueous dilutions of QAC that would kill a bacterial suspension in physiological saline within 10 min. and to determine the survivor curve for this reaction. This was done as follows: Four milliliters of an aqueous dilution of the QAC, 5/4 as strong as the desired test concentration, were placed in a large test tube. One milliliter of the test suspension having a density of 5×10^8 cells per ml. was added to the disinfectant and mixed. At one minute intervals, or more frequently if necessary, 1 ml. portions of the medication mixture were pipetted to 9 ml. portions of a QAC inhibitor (0.22% asolectin, 1.56% Tween 80 buffered at

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TABLE I

LOG COUNTS OF SURVIVOR CURVE DETERMINATIONS

	Time (min.)							
	0	1	2	3	4	5	6	7
1	8.40	7.76	6.04	4.38	3.40	2.70	2.00	1.30
2	8.42	7.66	6.26	4.58	3.40	3.23	2.54	2.46
3	8.11	7.38	6.42	4.97	3.86	3.20	2.56	1.30
4	7.99	7.23	6.18	4.97	4.18	3.26	2.52	1.81
5	8.28	7.49	5.99	4.92	3.95	3.60	2.23	1.54
Mean	8.24	7.50	6.20	4.76	3.76	3.20	2.37	1.68

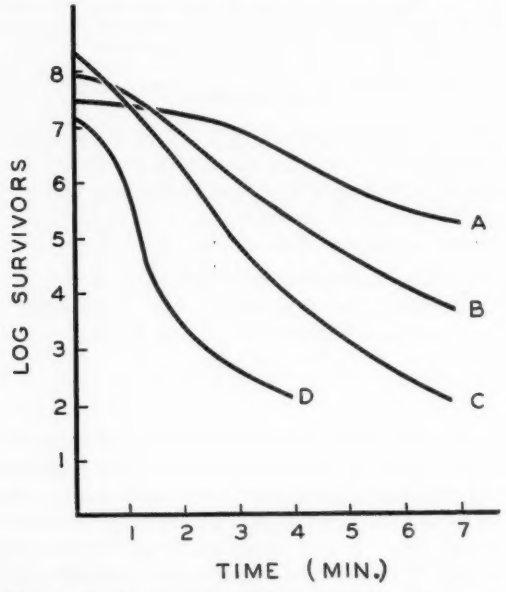

FIG. 1. Survivor curves. A. *Salmonella typhosa;* 25 p.p.m. Roccal. B. *Escherichia coli;* 40 p.p.m. Roccal. C. "Coliform"; 20 p.p.m. Roccal. D. *Serratia marcescens;* 50 p.p.m. Roccal. Room temperature.

pH 7.2 (16)) in stoppered tubes. The contents were shaken thoroughly and serially diluted for plating on tryptone glucose extract agar containing 0.1% asolectin and 0.7% Tween 80.

A coliform organism isolated from raw milk was used as a test organism for the first series of experiments. Twenty parts per million of Roccal was found to give a satisfactory end point (i.e. within 10 min.) when the final density of the suspension was 10^8 cells per ml.

Five replicates of this experiment are compared in Table I which shows the log counts of survivors during seven minutes of test and the log average values for each time interval. Plotting these results as log of survivors against time gives a sigmoid curve. Similar curves were found with *Escherichia coli*, *Salmonella typhosa*, and *Serratia marcescens* in the presence of several QAC (Fig. 1).

Migaki and McCulloch (12) investigated the possibility that "exhaustion" of the disinfectant would permit the constant decrease of the death rate observed in their studies, but were unable to show it at the concentration of disinfectant used. This was done by adding a second and a third inoculum when the phase of rapid death of the preceding inoculum had neared the end. This experiment was repeated in the present study and the result is to be seen in Fig. 2. The coliform organism and 20 p.p.m. Roccal were used. It can be

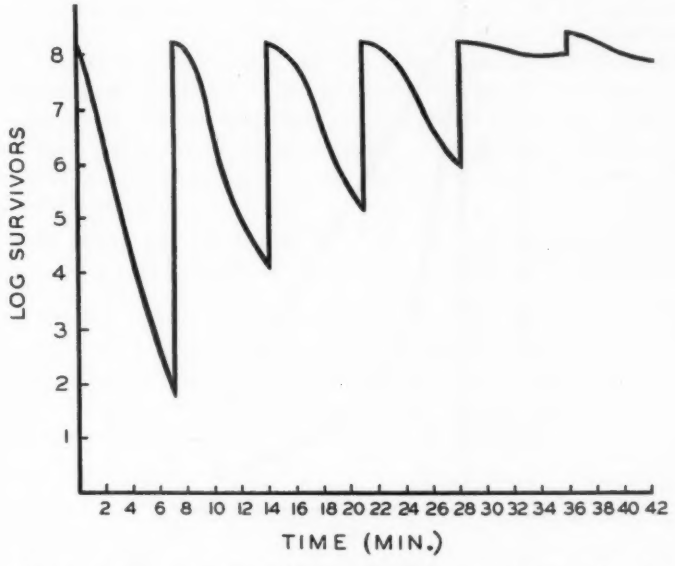

FIG. 2. Exhaustion of disinfectant. Five subsequent inocula added to original medication mixture. Room temperature.

seen by this sequence of curves that eventual exhaustion of the disinfectant occurs but that this would not explain the tapering off of the curve of the first inoculum.

Wide variation among the cells of the test suspension with regard to their resistance to the killing action of the quaternary compound could explain the sigmoid survivor curve. If this is so, it should be possible to select a more resistant strain by propagating the last survivors of the disinfectant action.

This was done by treating a test suspension of *S. marcescens* with 80 p.p.m. Roccal in the same manner used in the survivor curve studies, and at 20-sec. intervals transferring a loopful of the mixture to a subculture medium in which QAC inhibitor had been incorporated. The tube showing growth at 24 hr. representing survival for the greatest time of disinfection was selected for transfer to a nutrient agar slope. The 24-hr. growth was washed from the agar to prepare a suspension for a repetition of the same procedure. This whole process was repeated as many times as was necessary to eliminate all skips before the end point.

The survivor curve of the strain prepared in this way is compared with that of the parent strain in Fig. 3 and indicates that the normal strain is less resistant than the modified and that the latter now shows a logarithmic order of death.

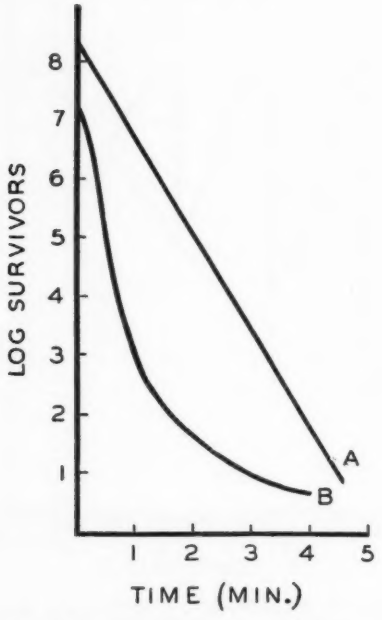

FIG. 3. Survivor curves; *Serratia marcescens*. A. "Selected" strain 60 p.p.m. Hyamine 1622. B. Normal strain; 60 p.p.m. Hyamine 1622. Room temperature.

In order to maintain the resistance to the quaternary necessary to show the logarithmic order of death of the modified strain it was found necessary to subject the strain constantly to the selection process outlined above. Passage on QAC-free media permits almost immediate reversal to the characteristics exhibited by the original strain.

Detergent/Protein Ratio

Klotz (9) showed that the degree to which QAC would react with protein depends upon the molecular weight of the detergent, and Putnam (14) proved that this reaction takes place only in proportions of detergent–protein equivalence. These observations are important in that nutrient broth as the substrate of a test suspension will react to an unpredictable extent with different QAC, and that if the action that is presumed to occur when detergent–protein equivalence prevails is presumed to occur also with bacterial cells as the protein (10) it is theoretically possible to provide an excess of the disinfectant in the presence of which cell death will not occur.

Substrate Protein

A measure of the QAC reaction with substrate protein was obtained in the following way: tubes containing 9 ml. of sterile nutrient broth were prepared, and to these known amounts of Roccal were added so that the final concentration of the QAC was not greater than 1200 p.p.m. The tubes were allowed to stand two days by which time the precipitate, which formed immediately upon the addition of the QAC, had adhered firmly to the tube walls. The QAC remaining in solution was titrated by the method of Harper, Elliker, and Moseley (6). Titrations of similar concentrations of QAC in distilled water showed that adsorption of QAC by the tube walls was not measurable by the titration method.

Table II shows the amount of QAC measureable by titration and, by difference, the amount of Roccal precipitated by the broth constituents.

TABLE II

PRECIPITATION OF ROCCAL BY NUTRIENT BROTH

Original conc. (p.p.m.)	Titrable after 48 hr. (p.p.m.)	Per cent precipitated
26	30	0
51	50	0
75	60	20
96	70	31
120	70	42
200	120	40
510	370	28
750	480	35
960	630	35
1200	750	37

Sterile 10% skim milk (Table III) treated in the same way separated into a curd and a clear supernatant liquid. The amount of Roccal precipitated is shown in the third column. Much of the lost QAC could be recovered by shaking, Column 5, but heating restored the QAC to the coagulum, Column 7. The maximum amount of Roccal precipitated is about 70%. Following

TABLE II

PRECIPITATION OF ROCCAL BY SKIM MILK

Original conc. (p.p.m.)	Supernatant titrable after 48 hr. (p.p.m.)	Per cent precipitated	Mixture shaken		Mixture heated	
			Titrable (p.p.m.)	Per cent pptd.	Titrable (p.p.m.)	Per cent pptd.
26	30	0	30	0	30	0
51	50	0	50	0	50	0
75	80	0	80	0	80	0
96	100	0	100	0	90	10
120	120	0	120	0	120	0
260	180	31	260	0	230	11
510	130	74	510	0	130	74
750	200	73	750	0	250	67
1060	230	66	680	21	300	69
1200	370	69	700	42	500	58

similar treatment four other QAC showed maxima of 70, 70, 65, and 45%. These results emphasize the inadvisability of conducting disinfectant tests in the presence of substrate protein.

Bacterial Protein

If, as Knox *et al.* (10) claim, the germicidal action of the QAC is dependent upon the detergent/protein ratio, it should be possible to demonstrate this by survivor curve studies based upon appropriate proportions of disinfectant and bacterial cells.

Survivor curves were, therefore, determined to observe the influence of the detergent/protein ratio on the death rate. Three curves were found, first, with the usual suspension and disinfectant dilution, second with each of these diluted one-half, and third, with each diluted one-quarter. The results are presented graphically in Fig. 4 and show a reduced death rate with reduced disinfectant concentration when the detergent/protein ratio is kept constant throughout.

Resistance to QAC

Bacteriostatic levels of QAC vary with the species. *M. pyogenes* var. *aureus* thrives in about 0.5 p.p.m. Roccal, *E. coli* in about 5 p.p.m., and *S. marcescens* in 50 to 100 p.p.m. when the disinfectant is incorporated in nutrient broth. These concentrations represent the amount of QAC added to the broth but do not necessarily represent that available to react with the cells since reaction with the beef extract of the broth may have intervened. Peptone does not form a precipitate with the QAC.

The possibility that increased resistance to the disinfectant may eventuate is one that deserves consideration particularly when the QAC is to be used repeatedly for sanitization. To determine the extent possible, *S. marcescens*

in a nutrient broth culture was transferred to a series of broth subcultures containing increasing concentrations of Roccal. After 24 hr. incubation at room temperature, transfers were made from the tube showing growth in the presence of the highest concentration to tubes with still higher concentrations.

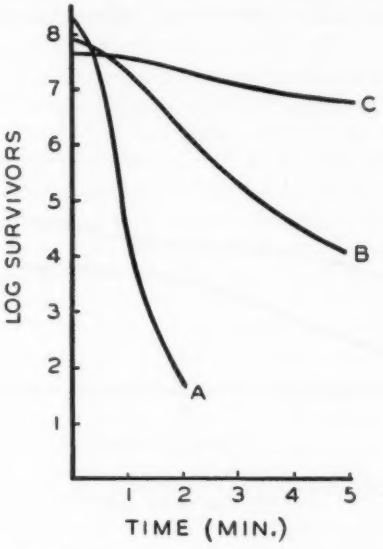

FIG. 4. Survivor curves at constant detergent/protein ratio. A. Roccal 30 p.p.m.; *E. coli* 1.9×10^8 cells per ml. B. Solution and suspension of A diluted one-half. C. Solution and suspension of A diluted one-quarter.

The steps in this process are presented in Fig. 5 and show that *S. marcescens* could be induced to grow in 10,000 p.p.m. Roccal after only eight transfers. *E. coli* did not respond to the same extent, but its limit was raised from 5 p.p.m. to 217 p.p.m. in seven transfers (Fig. 5). Attempts to adapt *M. pyogenes* var. *aureus* to increased concentrations of QAC all ended in failure. Repetitions of these experiments with *S. marcescens* and *E. coli* showed that the rate of acquisition of resistance was not constant.

Acquisition of resistance to one QAC probably confers a group resistance. *S. marcescens* adapted to grow in nutrient broth with 1000 p.p.m. Hyamine 1622 was transferred to broth subcultures containing 1000 p.p.m. of 16 other QAC. Growth occurred in eight of these. Similarly a strain adapted to 1000 p.p.m. Roccal when transferred to an equivalent concentration of 20 other QAC produced growth in 16 of them.

Death of Spores

Manufacturers' claims regarding the sporocidal activity of the QAC, particularly when in the form of a detergent–sanitizer, have led to some use of these products in the food industry. Amosol, a detergent–sanitizer, was included in a group of QAC in a series of survivor curve studies on a spore suspension of *Bacillus stearothermophilus* (National Canners Association strain

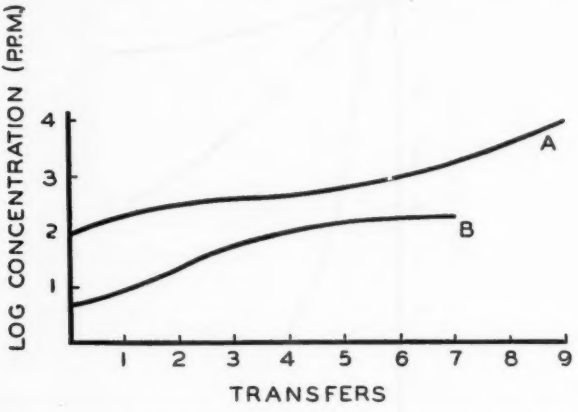

FIG. 5. Rate of acquisition of resistance. A. *Serratia marcescens*. B. *Escherichia coli*.

No. 1518, flat sour). Under the conditions of the experiment, which were identical with those described previously with the exception that the test suspension was heated to 80° C. for 10 min. before use, it was not possible to show that any killing had taken place in the presence of 2500 p.p.m. of the detergent–sanitizer. This is 100 times the concentration recommended by the manufacturer for complete killing of up to 10,000,000 spores per ml. in 10 min.

Discussion

The sigmoid order of death is an indication of a wide spread of resistance among the individual cells of a normal population. This belief was confirmed by isolating the more resistant portion of the population and observing the logarithmic order of death exhibited by this strain and its heightened resistance compared with the parent strain.

The detergent–protein complex formed by QAC and skim milk is relatively unstable. Much of the QAC so bound can be made available by shaking the mixture, but following heat treatments the complex is more firmly established. The bacterial death rate is not dependent upon the disinfectant–cell ratio, but, as is the case with other disinfectants, upon the concentration of

the disinfectant. If the loss of QAC efficiency in the presence of protein (7) is merely the result of the formation of a QAC–protein complex it should be possible to measure it directly by titrating the unbound QAC.

The resistance of bacterial cells to the action of QAC varies not only among species, but even among cells of the same suspension. For this reason an end point test such as the phenol coefficient is liable to be in great error depending on the element of chance in transferring resistant cells.

The isolation of a resistant strain by the selection of the last survivors of disinfection is of interest because of the lack of success in this attempt in the past (15). This was found to be more easily accomplished with *S. marcescens* than with *E. coli*, and was unsuccessful with *M. pyogenes* var. *aureus*.

In the selection of the resistant strain there is, of course, no adaptation. It was found possible to cause adaptation of *S. marcescens* and to somewhat less extent *E. coli* when these organisms were grown in increasing concentrations of QAC. The rate of acquisition of resistance (Fig. 5) is not exponential suggesting that the mechanism concerned is not the development of a single enzyme system (4, 13). Judging by the results obtained with these three species it would appear that the greater the initial resistance of the species the greater is the potential for acquiring higher resistance.

The absence of sporocidal activity recorded here and elsewhere (3, 5) is of interest in that the products recommended for sanitization of food packing plants do, apparently, perform this function satisfactorily. If contaminating spore formers present no problems following the use of detergent–sanitizers it matters little how or in what growth stage the organisms are destroyed. It is probable that the spore adsorbs sufficient QAC so that the young cell encounters a bactericidal concentration of QAC as the spore germinates. It is understood that most of the work upon which the manufacturers' claims are based was done before adequate information on QAC inhibitors was available.

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PHYSIOLOGICAL AND BIOCHEMICAL STUDIES IN PLANT METABOLISM

III. THE EFFECTS OF STARVATION AND MECHANICAL STIMULATION ON THE RESPIRATORY METABOLISM OF THE FIRST LEAF OF THE WHEAT PLANT[1]

By D. W. A. Roberts[2]

Abstract

The oxygen uptake and carbon dioxide output records of detached starving first leaves of wheat have been investigated. Small changes in the oxygen tension or carbon dioxide tension of the surrounding air do not make any difference in the records. The data obtained with the Barcroft method agree with those obtained by the Pettenkofer method. The form of the starvation air line is described. It is generally similar in the four quarters of the leaf but the senescent hump of the basal quarter is much flatter and its peak much lower than in the other three quarters. The total senescent hump carbon dioxide is shown to be proportional to the protein nitrogen in each of the four quarters. The effect on the air lines of rubbing and quartering the leaves is described. There is a great temporary increase in carbon dioxide output as a result of rubbing. This quickly subsides and the respiration rate after the rubbing settles down to a value slightly in excess of that prior to the rubbing. Rubbing and quartering have no effect on the R.Q. of the starving leaves.

Introduction

During their intensive studies of the starvation of the first leaf of the wheat plant Duff and Forward (7) observed that the color changes which take place during starvation do not occur simultaneously in all parts of the wheat leaf. Consequently an investigation into the physiological differences between the different parts of the leaves of wheat was begun. However, as a preliminary step in this investigation it was necessary to find out what the effects of starvation and handling were on the leaves. The present paper deals with these problems.

The publications of Audus (1, 2, 3) show that as a result of handling leaves the respiratory rates of the leaves may be increased greatly. This effect gradually wears off with the passage of time after handling. Since, in any determination of the respiratory rate of leaves, it is necessary to handle them, the magnitude of the effects of this treatment needs to be determined.

Because all determinations of respiratory rate were to be carried out on darkened leaves isolated from the plants, the effect of this isolation and starvation on the leaves also needed to be evaluated. A considerable amount of work on this phase of the respiratory metabolism of wheat leaves has been done previously in this laboratory (5, 7, 8). In almost all the earlier work whole leaves were studied and the respiratory rate was measured by the Pettenkofer

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A contribution from the Department of Botany, University of Toronto. An abridgement of part of a thesis presented in 1948 to the University of Toronto in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] *Plant Physiologist, Dominion Laboratory of Botany and Plant Pathology, Lethbridge, Alta.*

method. In the present studies the Barcroft differential manometer has been used, and the oxygen consumption of the quarters of wheat leaves has been determined. Consequently the results of the two methods had to be compared and reconciled. As will be shown these two methods give results which are in agreement with each other.

Materials and Methods

The plants used in these experiments were grown under controlled conditions. Seed of Khapli Emmer wheat (*Triticum dicoccum* Schrank. var. Khapli) obtained from the University of Saskatchewan, Department of Field Husbandry (their strain No. 681) was used. Only seed from the current crop year was used since it was found that seed from the preceding crop year was slower in germinating. This slower rate of germination is correlated with a slower rise in the rate of respiration in the first few hours of germination (10).

The wheat was grown under artificial conditions of comparative uniformity with a mean temperature close to 22.2° C., approximately 55% relative humidity, and a 12 hr. day of 500 ft-c. light produced by eight 500 w. incandescent light bulbs. Rich clayey garden soil, sifted and mixed with a little sand, leached cinders, and leaf mold was used. Forty seeds were planted in three concentric circles in waxed 1 lb. coffee tins with drainage, and covered with approximately one centimeter of soil. They were watered daily with 60 ml. of tap water except on Day 4, and all days after and including Day 11. On these latter occasions double strength mineral nutrient solution (Pfeffer's solution with added boron and manganese) was used.

On the ninth day, all plants whose first leaves did not lie between 11.5 cm. and 13.0 cm. were removed from the tins. The second leaves of the remaining plants were removed. Following this the second leaves and shoots were clipped every other day.

For the respiration experiments, the leaves were cut from the plants just below the ligule, and were placed in distilled water in diffuse light and subsequently weighed. In those cases where quarters were used, the leaves were individually quartered, the quarters all being of equal length. Samples were taken unless otherwise specified just after the close of the period of illumination.

Oxygen uptake of the leaf samples was determined by the Barcroft manometric method as described by Dixon (6). The data obtained by this method were compared with the results of carbon dioxide determinations by the Pettenkofer apparatus equipped with a Blackman automatic air-commutator arrangement.

The R.Q. determinations were made in the Barcroft apparatus by omitting the potassium hydroxide from the reaction flask. When the alkali is omitted from the reaction flask, the apparatus measures the difference between the oxygen uptake and carbon dioxide emitted. If then the oxygen uptake is known from a similar sample the R.Q. can be calculated. In this way the R.Q. can be followed continuously. One precaution is necessary. The flask

constants for oxygen and carbon dioxide must be very nearly equal to each other when calculated from the unsimplified formula given by Dixon (6). To achieve this the volume of water used in the flasks must be quite small relative to the total volume of the flasks.

The plants used for the determination of soluble and protein nitrogen (actually nondiffusable nitrogenous matter coagulated by boiling water) were treated in the same way as the samples for respiration studies except that the leaves were approximately quartered instead of being individually and exactly quartered. The methods used for nitrogen determination and the fractionation of the nitrogen have been described earlier (9).

The Effects of Changed Oxygen and Carbon Dioxide Tensions on the Rate of Respiration

The respiration rates of some tissues of higher plants are known to be affected by the oxygen tension and carbon dioxide tension in the surrounding atmosphere. In the experiments reported in this paper both oxygen and carbon dioxide tensions change during the course of the experiments. Consequently it is necessary to find out whether the changes which occur are great enough to interfere with the results obtained.

In the experiments to determine the rate of oxygen uptake, the carbon dixoide tension will always be quite low owing to the presence of potassium hydroxide in the wells of the reaction flasks. However the oxygen tension in the flasks will gradually fall, especially when the experiment is of long duration.

To investigate the effect of lowered oxygen tension on the rate of oxygen uptake large samples of leaves were used. In the two experiments for which data are presented in Table I 30 mature leaves weighing between 2 and 3 gm. were employed in order to secure a rapid decrease in oxygen concentration. In Expt. 1 whole leaves were used, while in Expt. 2 quarters were used. After the experiment had been in progress for some time, the flask containing the leaves was removed from the manometer. A tube was inserted into the flask and the air was sucked out of the flask in order to restore the oxygen concentration to that of the atmosphere. The flask was then replaced on the manometer, and the experiment continued. An examination of the results given in Table I shows that the reduction in oxygen concentration caused by this treatment does not affect the respiration rate significantly. Calculations showed that the oxygen concentration in the first experiment was reduced to 17.06% by volume, and in the second experiment to 15.2% by volume. These results are in agreement with those of Brodie (5) who found no difference between the respiration rate at 21% oxygen and 17% oxygen.

In the case of changes in carbon dioxide concentration in the atmosphere surrounding the leaves, no experiments were performed to test the effect of these changes. However the data presented in Table III indicate that within the limits of the experiments described in this paper the changes in carbon dioxide tension in the respiration flask do not affect the progress of the respiration record. In these experiments dealing with the effect of stimulation on

TABLE I

THE EFFECTS OF REDUCED OXYGEN CONCENTRATION CAUSED BY OXYGEN UPTAKE OF LEAVES WITHIN THE FLASKS ON THE RATE OF OXYGEN UPTAKE BY WHEAT LEAVES

Expt. 1		Expt. 2	
Time, min.	Oxygen uptake, mgm. per 100 gm. fresh wt. per hr.	Time, min.	Oxygen uptake, mgm. per 100 gm. fresh wt. per hr.
0 – 10	30.4	0 – 30*	52.0
10 – 70	27.4	30 – 65*	44.0
70 – 80*	24.0	65 – 135*	38.8
80 – 115	22.7	135 – 180	33.1
115 – 130	21.0	180 – 210*	30.7
130 – 145	20.4	210 – 240	28.3
145 – 160	20.5	240 – 270	27.5
160 – 175*	18.4	270 – 300	27.1
175 – 190	18.8	300 – 320	**
190 – 205	18.0	320 – 330	24.5
205 – 220	18.4	330 – 400	24.9
220 – 235	17.7	400 – 430	25.2
235 – 250	15.7	430 – 500	25.2
250 – 265	16.5		
265 – 280	18.6		
280 – 300	**		
300 – 310	25.6		
310 – 320	16.9		
320 – 330	15.5		

* *Taps on Barcroft manometers opened.*

** *Flasks for Expts. 1 and 2 opened during the time intervals indicated. Expts. started again at the time indicated. The opening of the flasks at these two times has been used to restore the oxygen concentration within the flasks to that in the atmosphere.*

the R.Q. two experiments were performed in which the leaves had a long sojourn in the respiration flask before stimulation. In both cases the effect of stimulation was normal and no changes in the R.Q. were observed. So the conclusion can be drawn that small increases in the carbon dioxide tension do not cause changes in the R.Q. of the leaves.

The Effects of Isolation and Starvation

In the earlier studies on the respiration record of starving isolated wheat leaves (5, 7, 8), air lines similar to the reconstructed one shown in Fig. 1 were obtained. Examining this figure it can be seen that the first phase is an approximately exponential decline in the rate of carbon dioxide output which swings into a second rising phase after 24 hr. According to Brodie (5) the R.Q. falls at this time from 1.0 to 0.8. After the 60th hour the third phase starts and the respiration rate again falls until disorganization and death occur after about 110 hr. Typically there are three sudden changes in the slope of the air line, one in each of the three phases described above. These changes of slope have been referred to as transpositions (7). Only the first and third transposition show clearly in the record of Fig. 1.

Using the Barcroft method a rough check on the respiration record of greenhouse grown wheat leaves was made. In this experiment, Table V, the same three phases described above were found, but, since only average

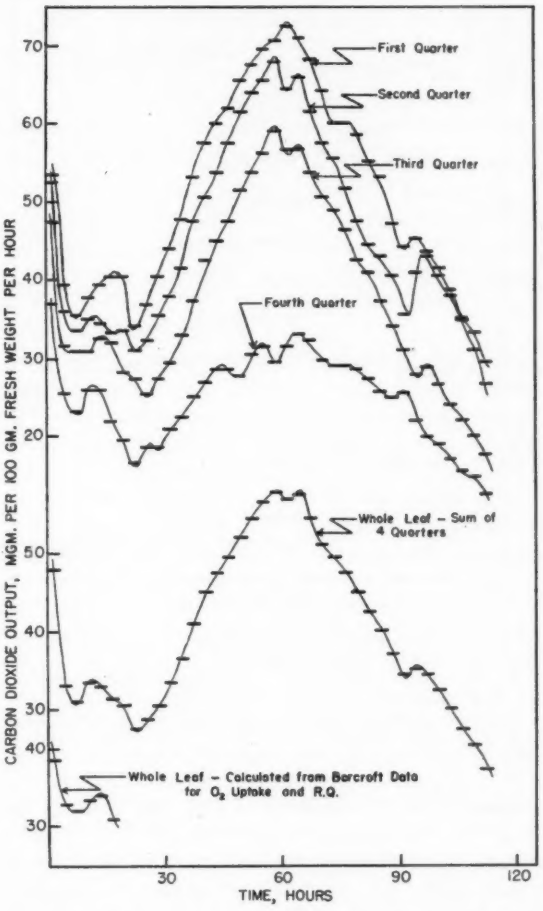

FIG. 1. The respiration records of the quarters, and whole first leaf of Khapli wheat as determined by the Pettenkofer method.

values could be obtained for the long overnight periods when the transpositions were expected to occur, these phenomena were not observed. There is, however, ample evidence that the first transposition is present in the air lines of leaves as determined by the Barcroft method.

In Fig. 2 the air lines of the first leaves of 13-day-old wheat plants are shown. These graphs give the oxygen uptakes as determined by the Barcroft method and do not appear at first sight to be similar to the Pettenkofer records of

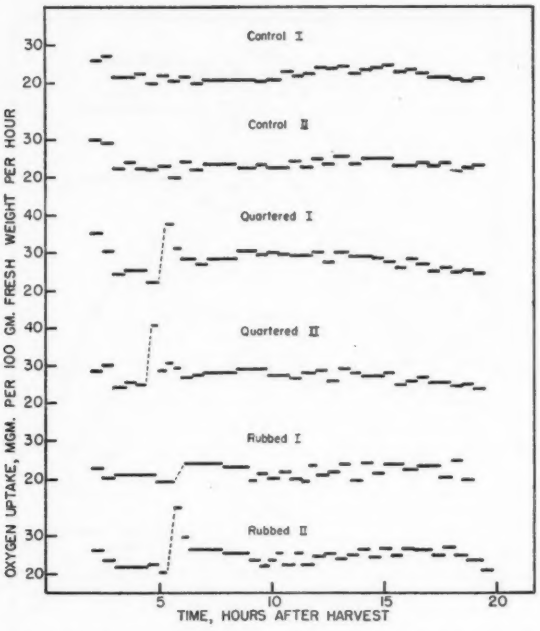

FIG. 2. The respiration records of standard first leaves of Khapli wheat by the Barcroft method.

"Control" samples remained in the respiration flasks throughout the experiment.

"Quartered" and "Rubbed" samples were removed from the flasks at the time indicated by the broken line, rubbed or quartered, and then replaced in the respiration flasks.

Fig. 1. This dissimilarity is largely due to the difference in the time scale used for plotting the record, and the fact that the R.Q. is not constant, as will be shown later. If we consider the air lines from Fig. 2 the following four phases can be identified: (*a*) a stimulatory decline lasting about two hours, (*b*) a level phase lasting about six hours, (*c*) a transpositional hump lasting about six hours, (*d*) a declining phase.

The reason for considering that the initial rapid decline in respiration is due to handling the leaves will be quite apparent from the ensuing account of the effects of rubbing and quartering the leaves. Its duration varies considerably and may be as short as one hour or as long as four hours.

The duration of the level phase varies greatly. Since the transpositional hump usually appears, unless obscured by stimulatory effects, about 10 to

12 hr. after the close of the period of illumination, it follows that the duration of the level phase will be controlled by the time at which the leaves are gathered.

The transpositional hump is rarely shown very distinctly by whole leaves. It is however, quite sharp and well developed in the records of the upper two quarters. In the Pettenkofer air line for the tip quarter shown in Fig. 1 there is a very well developed transpositional hump. The Pettenkofer records of Fig. 1 differ from most of the Barcroft respiration records that have been obtained in that it shows a transpositional hump in all four quarters.

The declining phase does not show fully in any of the Barcroft records, and the few data available suggest that it is not well marked in oxygen uptake records. The start of this phase shows clearly in the records labelled Control I and Quartered I in Fig. 2. Its duration is approximately six hours and it terminates, in the case of standard leaves, about 24 hr. after the close of the preceding period of illumination. It is shown very clearly in the basal quarter in the Pettenkofer experiment of Fig. 1.

In Fig. 3 are shown the air lines for the quarters of 13-day-old standard first leaves of Khapli wheat, together with a reconstruction of the air line for the whole leaf. This reconstruction was obtained by adding the oxygen uptakes from the four quarters together. This reconstructed record for the whole leaf is similar to the whole leaf records given in Fig. 2. The air lines for the four quarters are generally similar. The initial declining phase is seen to last longer in the basal or fourth quarter than in the other quarters. The possibility that this is due to differences between the stimulatory responses of the four quarters will be considered later. The transpositional hump is seen to be much more pronounced in the tip quarter than in the basal quarter. In the Barcroft records it has been usual to find little or no transpositional hump in the basal quarter (cf. however the Pettenkofer record of Fig. 1).

In order to follow the respiration record of the quarters throughout the whole period of starvation the Pettenkofer method was used. In these experiments the leaves were quartered and the quarters placed in groups of 20 in little cups so that their basal ends were standing in a little water. By using a parallel set of quarters it was possible to follow the color changes in the leaves without disturbing the respiration sample.

Prior to describing the air lines of these quarters which were harvested after 16 hr. of illumination, the color changes accompanying starvation will be considered. Even after 48 hr., there was no yellowing of any of the quarters, but paling of the first quarters near the base, and paling of the second quarters were evident. After 65 hr., the tip quarters were nearly all green-yellow with pale green tips (2–10 mm.) and cut basal ends (1–3 mm.). The second quarters were similar but there was no pale green band at the upper cut end. The yellowing in the third quarters was not so far advanced. In some of these quarters the green-yellow region occupied only a central band 5–10 mm. long while the others were yellow-green. Most of the basal quarters were

pale green but a few were as far advanced as the least advanced samples of the third quarters. At this stage, the color changes progressed rapidly. By the 90th hour, the tip quarters were yellow except for small pale green areas at

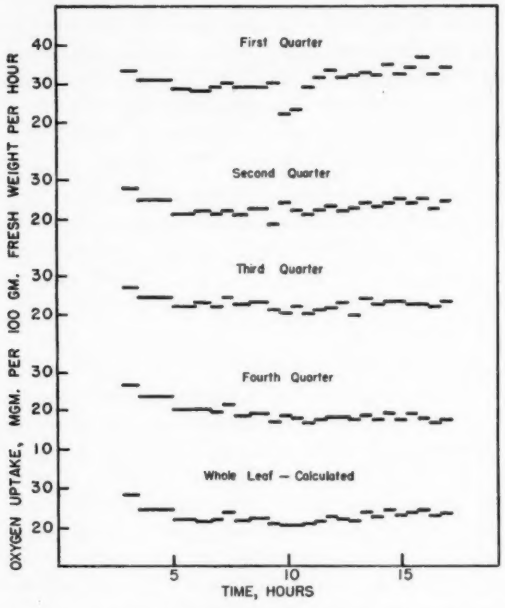

FIG. 3. The respiration records of standard quarters of the first leaf of Khapli wheat harvested after 12 hr. of illumination.

the tip and base. The second and third quarters were similar to the first except that they lacked the pale green tips. In the fourth quarters, most of the specimens were pale greenish yellow; but some had yellow central regions. By the 115th hour the browning of the tip quarter was well advanced, injection at the bases of the leaves was well begun, the leaves were soft and easily bruised, but many of the leaves retained a tinge of green at the tips and bases. The second and third quarters were similar, although the third quarters did not seem to be as far advanced as the other two. The fourth quarters were less far advanced in the color changes, more turgid, and just beginning to soften. Most of the leaves were yellow or white and only one showed any injection. At this stage the experiment was stopped.

Fig. 1 shows the results of the Pettenkofer experiment on the quarters, together with a reconstructed record of the whole leaves determined by adding the carbon dioxide emissions of the quarters together, and dividing by the combined weights. This record for whole leaves is typical of the results

which have usually been obtained by Duff and Forward for whole leaves except that the initial reading is higher than the usual value of about 40 mgm. of carbon dioxide per 100 gm. fresh weight per hour, and the second transposition is absent. The high initial rate is probably the result of the extra stimulation induced by quartering.

In Fig. 1 it will be seen that the record for the three tip quarters is very similar to that of whole leaves. The basal quarter shows a much more flattened senescent hump. Like the Barcroft records, these air lines show that the transpositional hump is most pronounced in the tip quarter and least developed in the basal quarter. It is also evident that the peak value for the senescent hump is greatest in the tip quarter, and becomes progressively less as the basal quarter is approached. A similar thing was observed in the Barcroft record of the greenhouse grown leaves mentioned earlier.

Brodie (5) came to the conclusion that most of the carbon dioxide of the senescent hump in wheat leaf starvation records was produced by the oxidation of protein degradation products. This conclusion has been tested with regard to the quarters. An examination of the protein distribution in the quarters of 13-day-old first leaves of wheat shows that there is a pronounced gradient in protein nitrogen from the tip of the leaves to the base of the leaves, with the most protein nitrogen in the tip quarter. Data given in Table II are the average values of protein nitrogen from a set of duplicate determinations. If, in the senescent hump, carbon dioxide comes chiefly from protein, then there should be a gradient in senescent hump carbon dioxide. The data in Fig. 1 and Table II clearly show that this is the case. The substantial constancy

TABLE II

THE RATIO OF SENESCENT HUMP CARBON DIOXIDE TO PROTEIN NITROGEN IN THE WHEAT LEAF QUARTERS

Quarter number	I	II	III	IV
Mgm. carbon dioxide per 100 gm. fresh weight from Pettenkofer experiments	4740 4890	4230 4550	3620 3750	2220 2630
Mgm. protein nitrogen per 100 gm. fresh weight	427	365	305	245
Mgm. carbon dioxide per mgm. protein nitrogen	11.1	11.6	11.9	9.1
	11.4	12.5	12.3	10.7

of the ratio between carbon dioxide and protein nitrogen supports Brodie's conclusion strongly. It is evident therefore, that protein distribution is responsible for the respiratory gradient in the leaves which is usually found during the phase of the senescent hump.

Before passing on to consider the comparison of the air lines of starving wheat leaves as determined by the Barcroft and Pettenkofer methods it is essential to consider the changes in the R.Q. of the leaves as starvation

proceeds. Using Duff's respiratory quotient apparatus, Brodie (5) reported that the R.Q. of wheat leaves having high sugar contents following 16 hr. of illumination was close to unity, and remained so for the greater part of the first 24 hr. after which time the R.Q. dropped rapidly to 0.8 at which value it remained throughout the greater part of the senescent hump. An examination of the results obtained with the Barcroft apparatus using the method described above gives a somewhat different picture of the drift of the R.Q. with starvation. The results are generally similar to those of Yemm (11) who worked with portions of barley leaves. The data in Table III and

TABLE III

THE EFFECT OF DELAYED STIMULATION ON THE RESPIRATORY QUOTIENT

Time, min. after darkening	Gas exchange mgm. per 100 gm. fresh wt. per hr. Expt. 1		Respiratory quotient	
	O_2	CO_2	Expt. 1	Expt. 2
120 – 240	25.4	37.5	1.08	1.10
240 – 305	20.6	30.5	1.07	1.03
305 – 365	18.6	26.3	1.03	1.06
365 – 425	19.7	28.3	1.04	1.04
425 – 465	15.4	21.8	1.03	1.12
465 – 535	20.0	29.1	1.06	1.07
535 – 575	17.6	24.8	1.03	1.04
575 – 610	17.7	24.6	1.01	1.04
610 – 640	19.8	26.8	0.99	1.00
640 – 670	18.5	25.3	1.00	1.02
670 – 700	17.5	23.8	0.99	0.99
700 – 730	18.9	26.0	1.00	1.00
730 – 760	19.5	26.8	1.00	1.01
760 – 790	19.5	26.8	1.01	1.02
790 – 820	21.0	28.8	1.00	0.99
820 – 855	18.9	26.9	1.03	1.04
855 – 880	21.0	28.8	1.00	1.04
880 – 910	20.8	27.5	0.96	0.96
910 – 940	19.2	26.0	0.98	0.96
940 – 970	19.4	26.0	0.97	0.99
970 – 985	*	*	*	*
985 – 1000	37.4	49.4	0.96	0.95
1000 – 1030	28.6	38.5	0.98	0.92
1030 – 1060	28.7	36.7	0.93	0.94
1060 – 1090	22.6	29.2	0.94	1.00
1090 – 1120	25.3	32.4	0.94	0.99
**				
1610 – 1670	25.2	32.3	0.93	0.86
1670 – 1730	26.5	30.7	0.85	0.83

* *The leaves were removed from the flask where respiration measurements were being carried out, rubbed, replaced in the flask, and the experiment restarted.*

** *The experiment was temporarily discontinued overnight.*

Fig. 4 show that the initial R.Q. is above unity and that the R.Q. tends to fall more or less continuously for the first 18 hours at least. There is definite evidence that during the period between Hour 10 and Hour 15 the R.Q. falls much more slowly or even not at all. At this time it usually lies close to

unity. The graphs suggest that, starting about Hour 18 after darkening the leaves, the R.Q. falls more rapidly again. The present experiments give values for the R.Q. 24 to 30 hr. after darkening of the order of 0.8 to 0.85 which agree with Brodie's figures.

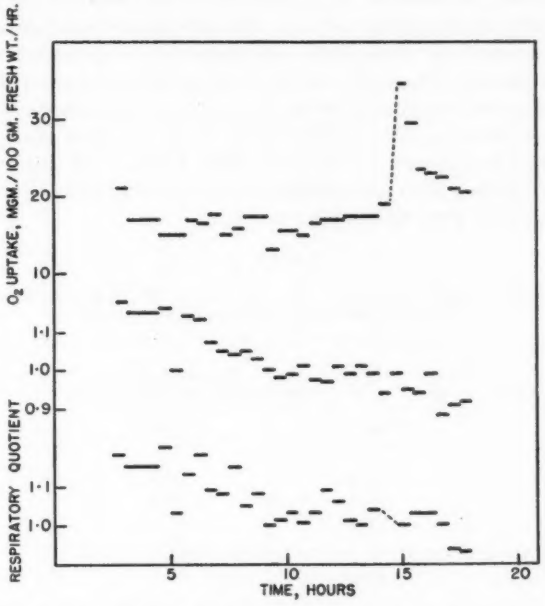

FIG. 4. The effect of starvation and stimulation on the records of R.Q. and oxygen uptake for the first leaf of Khapli wheat. The broken lines indicate the times when the leaves were removed from the flasks, rubbed, and replaced in the respiration flasks.

When the Barcroft air lines are compared with the Pettenkofer records there appear at first sight to be serious discrepancies between the two. However, a considerable part of these discrepancies disappears when the two are calculated on the same basis. Table IV presents a comparison of the Barcroft and Pettenkofer respiration values calculated on the basis of the same time scale. The first Barcroft value is rather lower than the corresponding Pettenkofer value but this may be due to stimulatory phenomena to be described shortly, or to the R.Q., which the typical Barcroft experiments show, is initially greater than unity. In Fig. 1 the data for Expt. 2, Table IV are plotted together with a typical Pettenkofer record obtained by adding together the values for the four quarters. In this case the Barcroft data have been corrected for R.Q. on the warrantable assumption that the typical Barcroft R.Q. values are applicable. In this connection it must be remembered that the initial value for the whole leaves given in the Pettenkofer

record is unusually high. Typical values for this datum are close to 40 mgm. carbon dioxide per 100 gm. fresh weight per hour. This record, reconstructed from the Barcroft data, is indistinguishable from a Pettenkofer record. If, however, attention is turned to the record of the greenhouse grown leaves (Table V), it will be seen that there is little or no evidence of the large drop in respiration rate which usually occurs in Pettenkofer air lines between Hours 15 and 24. Barcroft R.Q. data (Table III) show that a considerable drop in the R.Q. occurs between Hour 16 and Hour 25 of the starvation record. It is possible that during this time there is only a slight drop in the oxygen consumption but that owing to the rapidly falling R.Q. the rate of carbon dioxide emission drops much more rapidly and much farther. If this explanation is accepted then there are no appreciable differences between the Pettenkofer and Barcroft starvation records of wheat leaves.

TABLE IV

RATES OF CARBON DIOXIDE EMISSION FOR THREE-HOUR INTERVALS AS CALCULATED FROM BARCROFT DATA ASSUMING AN R.Q. OF UNITY

Time from start, hr.	Respiration rate, mgm. carbon dioxide per 100 gm. fresh wt. per hr.		
	Expt. 1†	Expt. 2††	Expt. 3†††
0 – 3	36.3	32.8	33.2
3 – 6	31.8	29.4	29.5
6 – 9	31.2	29.2	28.5
9 – 12	33.5	32.5	31.2
12 – 15	33.3	32.6	31.9
15 – 18	31.8	30.3	—

† *Calculated from the data plotted as "Control I", Fig. 2.*
†† *Calculated from the data plotted as "Control II", Fig. 2.*
††† *Calculated from the whole leaf record plotted in Fig. 3.*

One further point needs considering in connection with the respiration records of the starving leaves. This is the problem of the nomenclature of the different phases shown by the two types of records. The broad outlines of the two types are so similar that no difficulties arise in this connection. When the transposition of the Pettenkofer records is interpreted in terms of the Barcroft records, difficulties arise. If attention is turned to Fig. 1 where an imaginary Pettenkofer record has been constructed from the oxygen uptake, and R.Q. data obtained by the Barcroft method, then the equivalence of the different records becomes evident. Turning back to the actual Barcroft oxygen uptake records (Fig. 2), it is seen that the first point of the reconstructed Pettenkofer record corresponds to the stimulatory decline. Thus the very steep initial part of the Pettenkofer records of mature leaves is probably the result of handling the leaves. The second and third points on this reconstructed record are found to represent the level phase of the Barcroft record. Part of the flatness has disappeared, owing to the slowly falling R.Q.

TABLE V

OXYGEN UPTAKE RECORD OF GREENHOUSE-GROWN FIRST LEAVES OF WHEAT GATHERED 11.15 A.M.

Time from start		Oxygen uptake in mgm. per 100 gm. fresh weight per hr. for time interval indicated			
Hr.	Min.	Quarter I	Quarter II	Quarter III	Quarter IV
0	0				
1	45	22.8	16.5	16.7	10.0
3	15	15.9	10.8	11.9	9.4
4	45	15.3	11.8	10.8	4.5
6	25	17.9	9.9	10.8	9.4
20	40	19.8	13.4	12.7	8.8
22	0	23.2	16.3	13.9	9.7
23	45	22.8	14.8	15.1	12.6
26	0	24.5	16.5	11.8	9.1
29	40	26.4	18.5	16.6	11.2
46	5	35.0	27.4	20.8	15.6
47	20	40.0	29.7	25.9	15.7
48	55	39.4	24.8	29.0	20.9
50	0	39.8	28.8	26.4	17.9
51	35	39.1	25.7	31.7	18.8
52	45	39.4	24.7	30.4	16.9
70	5	33.8	19.8	28.2	18.2
71	25	32.9	10.4	20.4	21.2
72	45	26.2	9.9	19.2	22.6
74	0	28.8	14.4	20.6	14.4
75	0	23.6	—	12.7	23.8
77	20	24.2	—	12.9	16.4

The fourth and fifth points of the reconstruction correspond to the transpositional hump of the Barcroft records. The sixth point corresponds to the declining phase of the Barcroft records. Now the question arises as to the part of the Barcroft record which corresponds to the transposition of the Pettenkofer records. In the case under consideration, it is apparent that the transpositional hump corresponds to the transposition of the Pettenkofer records. In some Pettenkofer records, the transposition appears as two or more nearly equal values in a steadily declining arm of the record. In this case, the transposition must correspond with that part of the level phase of the Barcroft records where the R.Q. is falling slowly or not at all. Thus it can be seen that the transposition of the Pettenkofer records is, or may be, associated with the level phase of the Barcroft records, or the transpositional hump of these records. The Barcroft records indicate that the transpositional hump occurs between 10 and 12 hr. after the close of the period of illumination. This same thing seems to be true of the almost flat part of the R.Q. record from the Barcroft experiments. The coincidence of these two phenomena, steady R.Q. values and the transpositional hump, probably explains the regular occurrence of the transposition in the Pettenkofer records after about 12 hr. of darkness, even though the transposition may represent two different phases of the Barcroft record.

Effects of Mechanical Stimulation

Three types of treatment were given to the leaves to find out whether they produced the same type of modifications in the respiration record as those reported by Audus (1, 2). These were rubbing, quartering, and change in air pressure followed by return to normal air pressure. In all these experiments, the respiration record of the untreated leaves was followed for two to three hours and then, except in the case of pressure change, the leaves were removed from the respiration flask, rubbed or quartered, and returned to the flask where the air line was again followed.

When Audus (1, 2) studied the effect of rubbing leaves, he found that a sudden jump in the rate of respiration immediately followed the rubbing. This was succeeded by a rapid decline in rate to a value slightly in excess of the normal air line value. The respiration record of the rubbed leaves then ran parallel to the normal air line for many hours but finally slowly settled down onto the "unstimulated" air line. Modifications of the above were found to occur in starving leaves which had gone into their senescent rise and also in a few plants such as bamboo. Wheat was not tested. The present set of experiments shows that Khapli wheat displays the typical behavior described above, at least until the time that its starvation has proceeded well into the transpositional hump. Some of the records of stimulated leaves do not show the transpositional hump, and experiments will be described which indicate a possible reason for this.

The two air lines in Fig. 2 labelled "Rubbed I" and "Rubbed II" show the effect of rubbing the leaves. The break in the record filled in by the broken line indicates the time when the leaves were removed from the flask and rubbed once between the fingers. "Rubbed II" shows the great rise in the respiration rate immediately following the handling of the leaves. These very high rates of oxygen uptake only persist for a very short time after the leaves are rubbed. The first air line does not show these very high values because the first time interval is so long that the average rate of oxygen uptake during this long interval is not excessively high. The next point of note is that the general level of the air line after stimulation is higher than it is before the leaves are rubbed. A comparison of the air lines immediately after the stimulation shows the rapid drop in rate of oxygen uptake that is characteristic of the first few hours of the "unstimulated" air lines. It is on the basis of this similarity that the first two to four hours of the Barcroft records are referred to as the stimulatory decline. However the changes in R.Q. which occur during these first few hours show that factors other than stimulation affect the early part of the respiration record of isolated leaves since R.Q. changes do not accompany stimulation. The early parts of these air lines are also affected by starvation and it is difficult to decide the extent of either stimulatory effects or starvation effects. The rapid decline in oxygen uptake suggests that the effect of stimulation is very important at this stage.

Fig. 5 and Table VI give the results of experiments in which the leaves were rubbed on two separate occasions. The second rubbing produced an effect similar to that of the first rubbing. There is a suggestion that the peak

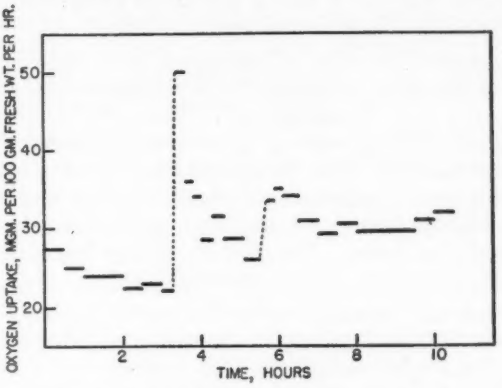

FIG. 5. The effect of repeated stimulation on the oxygen uptake of the first leaf of Khapli wheat. The leaves were rubbed at the times indicated by the broken lines.

TABLE VI

THE EFFECT OF RUBBING THE LEAVES ON TWO SEPARATE OCCASIONS

Time after darkening the leaves, min.	Oxygen uptake in mgm. per 100 gm. fresh wt. per hr.		
	Control†	Expt. 1††	Expt. 2††
180 – 210	22.0	20.9	24.2
210 – 245	19.3	19.5	22.8
245 – 305	19.1	19.3	23.0
305 – 315		*	*
305 – 375	17.0		
315 – 375		25.0	66.5
375 – 415	15.1	20.1	22.8
415 – 460	19.2	21.8	24.3
460 – 510	17.2	21.0	24.0
510 – 550	16.7	21.1	23.1
550 – 610	16.0	20.0	22.6
610 – 670	16.6	19.8	22.0
670 – 685		*	*
670 – 715	17.8		
685 – 715		21.5	26.2
715 – 745	16.8	22.3	25.6
745 – 775	22.1	27.8	30.0
775 – 805	18.6	23.8	25.8
805 – 835	17.7	22.0	22.4
835 – 865	18.5	23.4	26.9
865 – 900	18.2	23.6	23.3

† *Leaves not removed from flasks during experiment.*

†† *Leaves removed from the flasks, rubbed, replaced in flasks, and experiment restarted at times marked*.*

of oxygen uptake following the stimulation was lower after the second rubbing than after the first rubbing. However, all the experiments did not show this effect. Audus (3) reported a similar effect after the first stimulation. The second stimulation produces a further rise in the poststimulatory respiration rate similar to that produced by the first stimulation.

The experiments on the effect of quartering were carried out in the same way as those on the effect of rubbing. The results are shown in Fig. 2 as air lines "Quartered I" and "Quartered II". These graphs are very similar to those showing the effects of rubbing except that the transpositional hump is absent from both of them, whilst the rubbed leaves show at least a suggestion of it. Since the leaves quartered immediately upon removal from the plant show a transpositional hump it was decided to try to discover the cause of the disappearance of that hump. The air lines for the leaves quartered immediately on removal from the plant are shown in Fig. 3.

Before proceeding to consider the disappearance of the hump the possibility that the effects of quartering described above are really due to quartering and not to removal of the leaves from the flask must be considered. In order to investigate this possibility parallel experiments were run. In one set of experiments the leaves were removed from the flasks and then replaced in the flasks. In the other set the leaves were removed from the flasks, quartered, and then replaced in the flasks. In both sets of experiments the typical stimulatory response was shown. It is therefore impossible to be absolutely sure that quartering produces any change in the air line of wheat leaves. As mentioned earlier, stimulation causes an increase in the poststimulatory respiration rate. This increase can be determined from an examination of the air lines of samples of stimulated leaves. A comparison of the magnitude of this increase in the case of leaves only removed from the flasks, with those removed and quartered is given in Table VII which reveals that the increase in the general level of the air lines is greater in the case of the samples which are removed and quartered. This is the only evidence that the operation of quartering of itself causes a stimulatory response in the air line of the leaves.

TABLE VII

INCREASES IN THE AVERAGE VALUE OF OXYGEN UPTAKE IN MGM. PER 100 GM. FRESH WEIGHT PER HOUR RESULTING FROM REMOVAL OF LEAVES FROM FLASK AND REPLACEMENT IN THE FLASK COMPARED WITH THAT RESULTING FROM REMOVAL FROM FLASK, QUARTERING AND REPLACEMENT IN FLASK

Expt. No.	Increase in average oxygen uptake	
	Removed and quartered	Removed only
1	6.4	3.8
2	5.6	—
3	2.2	−1.3
4	2.2	1.5
5	4.4	2.1
6	—	3.8

This set of experiments indicates that the amount of handling that the leaves are given influences the value of the respiration in the level phase. Undoubtedly differences in the handling of the leaves are an important factor in the variability of the respiration rates of leaves which have been grown and harvested under similar conditions. It is also in all probability responsible for the very high initial respiration rates obtained in the Pettenkofer experiments on the respiratory rates of the quarters reported above.

It has already been pointed out that the transpositional hump is missing from the air lines "Quartered I" and "Quartered II" of Fig. 2 while air lines of leaves gathered after one hour of darkness and quartered immediately do show a transpositional hump. Leaves were harvested from the plants after varying periods of darkness and their air lines followed to find out whether the hump was present or not. In the course of other experiments samples were harvested shortly after the close of the period of illumination and stimulated by rubbing or quartering several hours later. The respiratory records of these samples were also examined for the transpositional hump. The results are brought together in Table VIII. These experiments show that if the leaves are

TABLE VIII

THE EFFECT OF THE TIME OF STIMULATION ON THE PRESENCE OF THE TRANSPOSITIONAL HUMP

Hours from darkening when manipulated by	Sampling	Rubbing	Quartering
0	Hump present		Hump present
1	" present		
2	" present		" present
3	" present	Hump absent	
4	" absent		" absent
5	" present	" absent	" absent
	" doubtful	" present	
6	" present		
7	" absent	" present	" present
8	" absent		
9		" doubtful	
10	" absent	" absent	

gathered, quartered, or rubbed within the first two or three hours after the close of the light period the hump shows in the records. It also shows in the records of leaves similarly treated after about six hours of darkness. Leaves treated eight or more hours after the close of their illumination period do not show the hump because it is masked by the rapidly declining rates of oxygen uptake following the treatment. Only a tentative explanation for the absence of the hump after about four hours of darkness can be offered. It is suggested that about five or six hours after the stimulation there is a rapid fall in the rate of oxygen uptake due to the passing of the stimulatory effect, (cf. the

results of Audus (1) but with a greatly reduced time scale), and that this drop masks the transpositional hump which normally occurs 10–12 hr. after the close of the period of illumination. The air lines "Rubbed I" and "Rubbed II" Fig. 2 strongly suggest that such a drop occurs about four hours after the stimulation. It can be seen in these two records that if this fall in the respiration rate had been delayed another hour or two, all traces of the transpositional hump would have been obliterated.

In the studies of the effects of air pressure changes on the leaves, they were subjected to the different treatments within the flasks. This procedure was made possible by connecting the filter pump to both the compensating flask and the reaction flask containing the leaves. No response was found in the case of leaves subjected to a pumping action at 18 cycles per minute for 30 min. in which the pressure varied through 40 cm. of mercury in each cycle, or at 6 cycles per minute for one hour with pressure variations of 18 cm. of mercury. These results are in agreement with those of Bonner and Wildman (4), who found that injecting spinach leaves by vacuum infiltration did not affect the respiration rate.

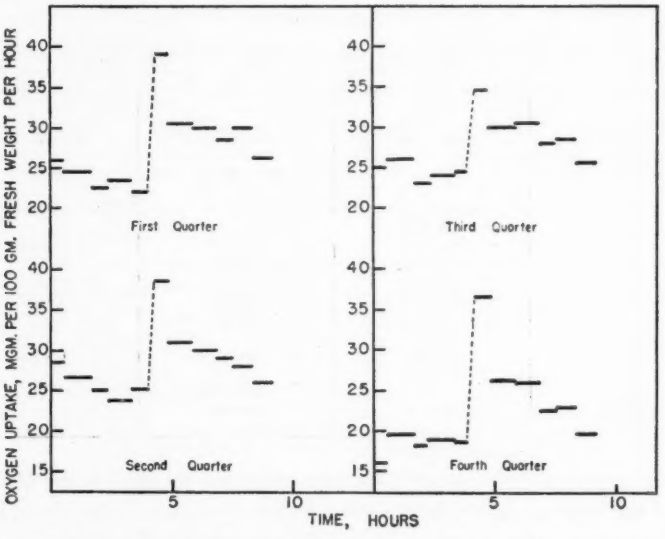

FIG. 6. The effect of stimulation on the four quarters of the first leaf of Khapli wheat. Stimulation was produced by rubbing after the removal of the leaves from the flasks. These operations were carried out during the periods indicated by the broken lines in the graphs.

In view of the effects of stimulation on whole leaves, it is pertinent to enquire whether quarters manifest the same phenomena, and whether the response to stimulation is the same in all the four quarters. Fig. 6 shows the effect of

rubbing the different quarters. The experimental procedure was the same as in the case of whole leaves, except that the leaves were quartered before the start of the experiment. The effects of stimulation on the four quarters are seen to be quite similar. There is a suggestion, however, that in the tip quarter, the respiratory rate falls to a plateau value sooner than in the other quarters, especially the basal quarter. After stimulation, the respiratory rate in the basal quarter falls rapidly at first, and then slowly for a considerable time, while in the tip quarter the rapid fall soon ends in a relatively steady phase. This same thing occurs in the early part of the records of "unstimulated quarters" and is probably due to the slight differences in the response of the different quarters to handling.

Since it has been shown that rubbing causes a great increase in the oxygen consumption, the question arises whether stimulation has any effect on the R.Q. of the leaves. Three experiments in which the leaves were rubbed a few hours after gathering suggested that stimulation produced no appreciable effect. In order to settle this point two experiments were performed in which the rubbing was delayed many hours after the start of the air line record. The results of one of these experiments is shown in Fig. 4 where the broken line indicates the period when the leaves were being removed from the flask, rubbed, and replaced in the flask. The upper graph shows that the leaves gave the typical response to rubbing as far as oxygen uptake is concerned. A comparison of the two lower graphs shows that the R.Q. record of untreated leaves (middle graph) is the same as that of rubbed leaves (bottom graph). The results of the other experiment, which are given in Table III, also show that stimulation has no effect on the respiratory quotient of wheat leaves.

Acknowledgments

The original problem was suggested by Prof. G. H. Duff who together with Prof. D. F. Forward offered many valuable criticisms and suggestions during the course of the research. Prof. G. H. Duff and D. F. Forward carried out the Pettenkofer experiments on the quarters of the wheat leaves. During these experiments they were assisted by Miss J. Scorgie.

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BIOCHEMISTRY OF THE USTILAGINALES

V. FACTORS AFFECTING THE FORMATION OF USTILAGIC ACID BY *USTILAGO ZEAE*

By J. A. Thorn[2] and R. H. Haskins[3]

Abstract

In a study of factors affecting the formation of ustilagic acid by *Ustilago zeae* (PRL 119) in submerged culture, yields of the acid ranging from 5 to 23 mgm. per ml. were obtained in three to five days, depending upon the concentration of medium constituents and the rate of aeration. The media employed contained cerelose, urea, corn steep liquor, and inorganic salts. Yields of ustilagic acid were usually between 8 and 15 mgm. per ml., corresponding to 12 to 23% conversion of the available glucose, on a carbon basis. Yields of the acid increased with increasing rates of aeration. Urea gave slightly higher yields than did the ammonium salts tested. Corn steep liquor concentration was optimal at 0.06%. Yields of ustilagic acid increased with increasing cerelose concentration, but the rate of conversion of carbohydrate into the acid decreased. About the same yields of ustilagic acid were obtained in shaken 500-ml. flasks, in nine-liter, and 30-liter fermentors.

Introduction

Ustilagic acid, a mixture of glycolipids (5, 6), is obtained when *Ustilago zeae* (PRL 119) is grown submerged in artificial media containing glucose as the source of carbon. The acid is formed under a wide variety of cultural conditions, only simple nutrients being required by the fungus. This paper will describe the chemical changes occurring during the fermentation of glucose by *U. zeae*, as well as experiments designed to determine the factors leading to maximal production of ustilagic acid. Fermentations conducted in shaken 500-ml. flasks, nine-liter glass, and 30-liter stainless steel fermentors will be discussed.

Since preliminary experiments showed that yields of the crystalline metabolic by-product were greater with glucose as the carbon source than with several other sugars tested, the present paper is limited to the studies in which glucose (or cerelose) was used as the carbon source.

Materials and Methods

Fermentation Techniques

Ustilago zeae (PRL 119) was used exclusively in the work to be described and was grown at 30° C. The organism was propagated by repeated subculture and mass inoculum used in an attempt to achieve physiologically homogeneous material.

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[2] *Biochemist.*

[3] *Mycologist.*

The standard salts mixture employed contained, in gm. per liter of distilled water: potassium dihydrogen phosphate, 1.0; magnesium sulphate heptahydrate, 0.4; and ferrous sulphate heptahydrate, 0.03.

The basal medium contained, in gm. per liter of standard salts mixture: cerelose (glucose monohydrate), 100; corn steep liquor, 0.6; urea, 1.2; and calcium carbonate, 1.7.

For inoculum purposes, *U. zeae* was continuously subcultured in the basal medium, being grown for four to five days in shaken 500-ml. Erlenmeyer flasks each containing 100 ml. of medium. The amount of inoculum employed was 5% by volume of the medium to be inoculated.

Unless otherwise specified, experimental fermentations were conducted in shaken flasks as above, each flask normally containing 100 ml. of medium. The basal medium was employed but was altered according to experiment. Broth samples required for chemical analyses were obtained by aseptic technique with sterile pipettes. Usually, 3 to 5 ml. samples sufficed.

Both inoculum and experimental flasks were agitated by means of a rotary shaker operating at 220 r.p.m. with a horizontal radius of motion of one inch.

Larger fermentors employed included nine-liter glass and 30-liter stainless steel fermentors. The nine-liter fermentors consisted of narrow-necked pyrex bottles fitted with mechanical stirrers. Aeration was obtained by passing sterile air through a porous glass disc situated near the bottom of each bottle. Each fermentor was charged with four liters of medium containing, in gm. per liter of the standard salts mixture: cerelose, 50; urea, 0.6; corn steep liquor, 0.6; and calcium carbonate, 1.7.

The 30-liter fermentors were similar to that described by Rivett, Peterson, and Johnson (8). Each fermentor was charged with 15 liters of medium containing, in gm. per liter of tap water: cerelose, 50; urea, 0.6; corn steep liquor, 0.6; potassium dihydrogen phosphate, 1.0; and calcium carbonate, 1.7. The urea and calcium carbonate were each sterilized separately in water and added to the sterile medium shortly before inoculation.

Analytical Procedures

Reducing sugars were determined by the method of Shaffer and Somogyi (9), using their reagent 50 with 5 gm. of potassium iodide.

Total carbohydrate was determined by the anthrone method of Dreywood (2, 7). Total soluble carbohydrate was determined by this method on samples of the clear supernatant liquor from centrifuged broth.

The pH of each broth sample was determined immediately after removal from the flask by means of a glass electrode pH meter.

Ammonia and urea were determined by the microdiffusion methods of Conway (1).

Total and soluble Kjeldahl nitrogen were determined in the usual manner by means of a micro-Kjeldahl method. The difference between the soluble

Kjeldahl nitrogen and the sum of urea and ammonia nitrogen is reported as soluble organic nitrogen.

Cell nitrogen was determined by subtracting the soluble nitrogen present at time of sampling from the soluble nitrogen present at the time of inoculation. Cell carbohydrate (as glucose) was determined by analysis by the anthrone method of cells washed with water and methanol.

Ustilagic acid in broth samples was estimated by multiplying the difference between the total anthrone glucose (corrected for cell carbohydrate) and the residual reducing sugar by 2.16, the factor being based on the fact that ustilagic acid contains two nonreducing glucose residues per mean molecular weight of 777 (6). The method of estimation was checked by comparing estimated yields of ustilagic acid with yields found upon isolation of the acid from fermentation broths, agreement within 6% being found (10).

Experimental and Results

Fermentation in Basal Medium

Fig. 1 shows the chemical changes which occurred when *Ustilago zeae* (PRL 119) was grown in the basal medium. Owing presumably to the heavy inoculum employed, no appreciable lag phase occurred. The urea nitrogen

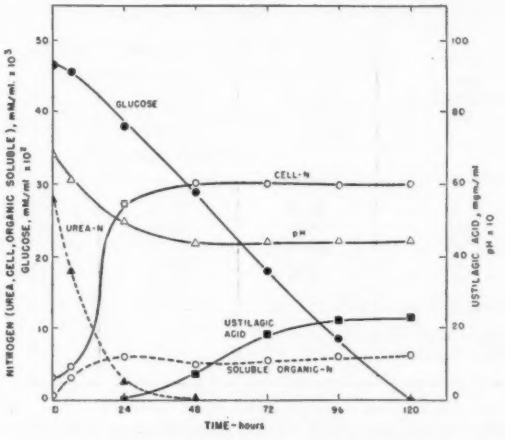

FIG. 1. Chemical changes occurring in the culture broth when *Ustilago zeae* (PRL 119) was grown in a 10% cerelose–nutrient medium in flasks shaken for five days at 30° C.

was rapidly assimilated and, in many of the fermentations conducted, it was found to be completely assimilated in the first 24 hr. of growth. The dissimilation of glucose was rapid and occurred at a nearly constant rate. The fermentation was characterized by an initial drop in pH, followed by a slow rise

beginning at approximately 48 hr. In media not containing calcium carbonate, the pH decreased rapidly to less than 4 in the first 48 hr., and the rate of glucose dissimilation became very slow.

Formation of ustilagic acid began usually between 24 and 48 hr. after the start of the fermentation, and crystals were apparent by 48 hr. The amount of ustilagic acid formed in the fermentation depicted in Fig. 1 (23 mgm. per ml.) was greater than that commonly obtained with this medium. Yields normally ranged between 10 and 15 mgm. per ml. Considerable differences in yields were occasionally found even when presumably identical conditions were employed during the fermentations. However, the yields were commonly quite reproducible.

Various factors which possibly could affect the extent and rate of formation of ustilagic acid by *U. zeae* were investigated. These are discussed below.

Effect of Aeration

Table I shows the effect of rate of aeration on the dissimilation of glucose and formation of ustilagic acid by *U. zeae*. The different amounts of basal medium were placed in 500-ml. Erlenmeyer flasks and, since foaming was not

TABLE I

EFFECT OF AERATION ON GLUCOSE DISSIMILATION AND USTILAGIC ACID FORMATION BY *U. zeae* (PRL 119)

Age of fermentation, hr.	Volume of medium in flasks		
	50 ml.	100 ml.	150 ml.
	Glucose, mgm./ml.		
0	88.0	91.2	92.8
24	61.6	78.6	83.5
48	20.8	61.2	71.4
72	0.2	45.0	58.5
96	0.1	25.5	48.6
Ustilagic acid at 96 hr., mgm./ml.	23	7	5

encountered, the rate of aeration of the medium was considered a function of its volume in the flasks, the greatest rate of aeration occurring in flasks containing least medium.

A marked difference in production of ustilagic acid was noticed also between fermentations conducted on the rotary shaker and on a reciprocal shaker which had a throw of four inches and operated at 65 cycles per minute. An experiment in which 50 ml. of basal medium per flask was used gave yields of acid of 19 and 8 mgm. per ml., respectively, in fermentations conducted on the rotary and reciprocal shakers.

In another experiment, sterile air saturated with water vapor was bubbled through the medium, while flasks with ordinary cotton plugs acted as controls. Each flask contained 100 ml. of basal medium and was agitated on the rotary shaker. At 96 hr. 99% of the glucose had been utilized in the aerated flasks as compared to 81% in the controls, and the corresponding yields of ustilagic acid were 14 and 9 mgm. per ml., respectively. Some foaming occurred in the aerated flasks.

Effect of Nitrogen

As stated earlier, urea nitrogen was rapidly utilized in the fermentation. In an effort to discover the effects of increased nitrogen concentrations upon ustilagic acid formation, media were employed containing 5 and 10 times the usual concentration (0.12%) of urea. However, at these higher urea levels, the media rapidly became alkaline and the fermentations failed.

Addition of urea at daily intervals was therefore attempted. The basal medium was employed but, at 24 and 48 hr., urea (as a sterile aqueous solution) was added to the fermentations in amounts roughly equivalent to that present in the medium before inoculation. Fig. 2 compares the chemical changes which occurred in these fermentations and those in the controls. As may be seen, the yields of ustilagic acid were the same in both cases.

Previous work (3) had shown urea to provide higher yields of crystals than potassium and ammonium nitrates. Table II shows a comparison of ustilagic acid yields obtained when four sources of nitrogen were employed in the fermentation medium. All four sources were about equally satisfactory although urea gave a somewhat higher yield.

As was the case with urea, the use of high concentrations of ammonium sulphate led to poor fermentations with concomitant low yields of ustilagic acid.

Effect of Corn Steep Liquor

A concentration of corn steep liquor of 0.06% appeared to be about optimal for ustilagic acid formation. Yields of the acid at 96 hr. were 14, 17, 15, and 9 mgm. per ml. when corn steep liquor was employed in the basal medium at concentrations of 0, 0.06, 0.12, and 0.30%, respectively.

Mineral Requirements

A simple study of the mineral requirements of *U. zeae* (PRL 119) already has been described (3). Since good growth of the organism occurred when only potassium dihydrogen phosphate and magnesium and ferrous sulphates were employed, further studies of the mineral requirements were not made. It was possible, of course, that impurities in the salts used were also required, but no indication of this was observed.

Effect of Cerelose Concentration

Table III shows the yields of ustilagic acid obtained when *U. zeae* was grown in the basal medium in which the cerelose concentration was varied from 2.5%

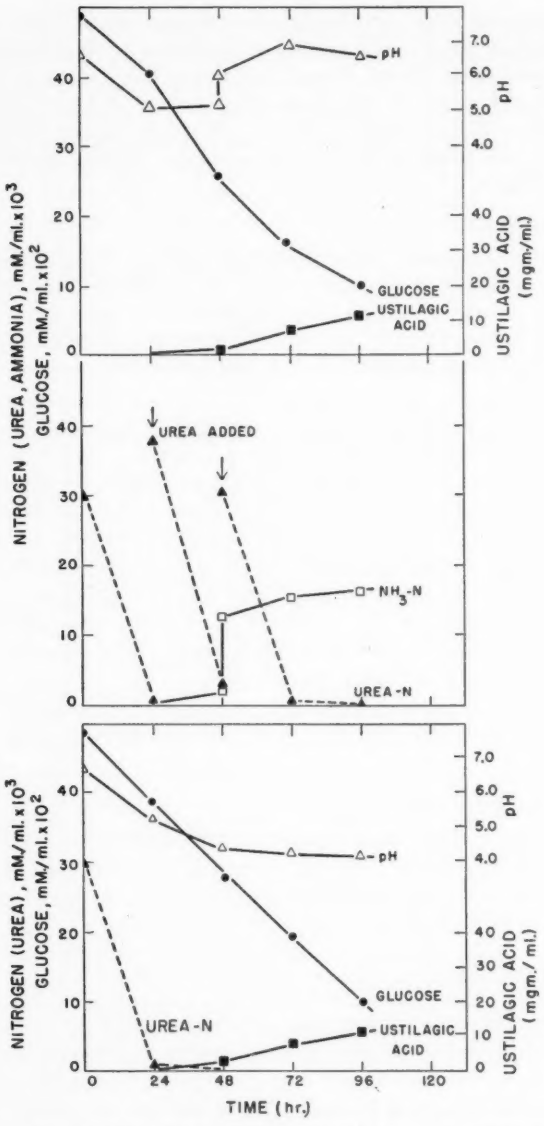

FIG. 2. Comparison of the chemical changes occurring during the fermentation by *Ustilago zeae* (PRL 119) of 10% cerelose–nutrient medium in two sets of flasks shaken for four days at 30° C. Urea was supplied to one set of flasks at 0, 24, and 48 hr. (top graphs) and only initially to the other set (bottom graph).

TABLE II

EFFECT OF DIFFERENT NITROGEN SOURCES ON USTILAGIC ACID FORMATION

Nitrogen source*	Ustilagic acid at 72 hr.,** mgm./ml.
Ammonium sulphate	11
Ammonium acetate	10
Ammonium lactate	9
Urea	12

* *Equivalent nitrogen levels used in each case.*
** *Averages of four flasks.*

TABLE III

RATE OF CONVERSION OF CERELOSE INTO USTILAGIC ACID BY *U. zeae* (PRL 119)

Initial cerelose, mgm./ml.	Length of fermentation, days	Yield of ustilagic acid		
		Mgm./ml.	Mgm./mgm. added cerelose	Mgm./mgm. added cerelose/day
25	3*	7	0.28	0.09
50	3	8	0.16	0.05
75	5	11	0.15	0.03
100	6	19	0.19	0.03

* *Dissimilation of the cerelose was very probably completed by the second day.*

to 10% and the ratio of cerelose to urea was held constant at 100 : 1.2. The broths were analyzed when less than 1% of the reducing sugar remained. Although yields of the acid (in mgm. per ml.) increased with increasing initial cerelose concentration, the rate of conversion of cerelose into ustilagic acid decreased.

Use of Larger Fermentors

Yields of ustilagic acid comparable to those obtained in shaken flasks were obtained in both nine-liter and 30-liter fermentors.

Six to seven days were required for complete utilization of the 5% cerelose in the nine-liter fermentors. This slow rate of fermentation was due possibly to limited aeration, but excessive foaming occurred when increased aeration was attempted. Yields of ustilagic acid ranged between 7 and 10 mgm. per ml.

Use of the 30-liter fermentors provided yields of ustilagic acid ranging from 10 to 21 mgm. per ml. Fermentations were completed in 44 to 72 hr. when the rate of stirring was between 200 and 500 r.p.m.; and the rate of air flow was between 0.24 and 0.30 liters of air per liter of medium per minute. Considerable foaming occurred, and lard oil was employed as an antifoam agent.

About 20 ml. of the oil, added in 5 ml. amounts as required, was found to be sufficient for each fermentation.

Discussion

The high yields of ustilagic acid in broth fermented by *Ustilago zeae* are particularly interesting. The average yields of 8 to 15 mgm. per ml. in media originally containing 10% cerelose correspond to conversion of available carbohydrate to the acid, on a carbon basis, of about 12% to 23%. The occasional higher yields of more than 20 mgm. per ml. represent conversions of greater than 30%. While other fungi have been reported to cause high conversions of carbohydrate to products (e.g. on a carbon basis, *A. niger* converted available glucose to citric acid in a 68% yield (11), and *Citromyces globium* gave a 30% yield of citromycetin (4)), few have been found to produce in such yields compounds as complex as ustilagic acid.

U. zeae has only simple nutritional requirements and grows well in aqueous media containing cerelose, urea, potassium dihydrogen phosphate, magnesium sulphate, ferrous sulphate, and calcium carbonate. The latter may not be needed from a nutritional viewpoint, but does act to control the acidity formed on dissimilation of the carbohydrate. The fungus is a quite rapid fermenter being able to utilize, under easily obtainable conditions, 10% cerelose in less than 120 hr. and 5% cerelose in less than 48 hr.

These characteristics of the fermentation, in addition to the antibiotic ability of the organism, will facilitate the operation of the large scale fermentations should any of the by-products of the fermentation prove of economic value.

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STUDIES OF VIRUS DISEASES OF STRAWBERRIES IN BRITISH COLUMBIA

II. THE SEPARATION OF THE COMPONENT VIRUSES OF YELLOWS[1]

By Frances C. Mellor and Randal E. Fitzpatrick[2]

Abstract

Progressive transfers of *Capilophorus fragaefolii* Ckll. from yellows-infected Marshall plants through a succession of *Fragaria vesca* L. plants resulted in the separation of at least two component viruses. One was of the nonpersistent type; it caused reduction in leaf size, mottle, crinkle, and leaf distortion, but the severity and dominance of these symptoms varied so widely that it is assumed that this component itself is a complex of viruses or strains. The other was of the persistent type; its principal effect was to reduce the vigor of the plant although there was some cupping of the leaves, and in the fall, under greenhouse conditions, a transitory yellow mottling of the tips and margins of some of the younger leaves.

Introduction

It has been shown by Prentice and Harris (4) and Prentice (2 and 3) that severe yellow-edge of strawberry as it occurs in England on the Royal Sovereign variety is the result of multiple infection by two or more separate viruses, any of which, alone, produces different and milder symptoms on the indicator plant *Fragaria vesca* L. Separation of the component viruses was achieved by varying the acquisition feeding period of the aphid vector on the diseased material and then transferring the insects to a succession of healthy plants.

Three viruses or groups of viruses were isolated, and designated by the authors as: virus 1 (mild crinkle virus), virus 2 (mild yellow-edge virus), and virus 3 (undesignated, but producing a more severe type of crinkle than virus 1).

On Royal Sovereign, virus 2 combined with virus 1, virus 3, or both, produced severe yellow-edge, whereas the combination of viruses 1 and 3 produced "a mild form of severe crinkle".

Strawberry yellows (xanthosis) of the Pacific Coast has much the same effect on the Marshall strawberry as that of yellow-edge on the Royal Sovereign. The symptoms induced on *F. vesca* by grafting to yellows-infected Marshall plants have been described by Fitzpatrick and Mellor (1); they are essentially the same as those produced by yellow-edge.

The experiments reported in this paper were designed to determine whether yellows, like yellow-edge, is the result of multiple infection by viruses that can be separated by techniques similar to those used by Prentice and Harris, and whether the component viruses correspond to the viruses found in England.

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Contribution No. 1100 from the Division of Botany and Plant Pathology Science Service Department of Agriculture, Ottawa.

[2] *Assistant Plant Pathologist and Plant Pathologist, respectively, Dominion Laboratory of Plant Pathology, University of British Columbia, Vancouver, B.C.*

Experimental

Material and Method

The source of inoculum was a clone of Marshall strawberry plants severely affected with yellows. These plants originated as runners from the plant used in the grafting experiments previously described (1).

The stock aphids (*Capitophorus fragaefolii* Ckll.) were hatched from eggs brought into the laboratory in the spring, and maintained on British Sovereign plants. When a population was required for a specific experiment, adult aphids were transferred to a fresh leaf where they were allowed to remain long enough to produce the required number of young and were then removed. When the young were three or four days old, they were transferred to the leaf which was to serve as the source of inoculum and allowed to feed for the length of time required in the particular experiment. In this way aphids of approximately the same age were used for each experiment and there was less chance, in subsequent transfers, of confusion between the original viruliferous insects and their nonviruliferous progeny. For the production of young for a specific experiment and for the acquisition feed, it was found convenient to keep the aphids on a detached leaf in a Petri dish. The stem of the leaf was placed in a vial small enough to retain water by surface tension when the vial was horizontal. During the transfer feeding period, the aphids were confined in a small cage consisting of a cork ring covered with fine linen. The cage was held in place on the under side of the leaflet by three pins, which went through the leaf blade into a cork disk or ring on the upper side (Fig. 1).

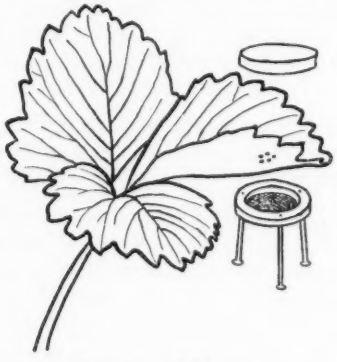


Fig. 1. Aphid cage.

Although the cages were very small, they would accommodate five aphids and all the progeny they produced in three weeks. However, in actual practice, it was found expedient to transfer the original aphids to a fresh part of the plant every 8 to 10 days and discard the young insects, for, if they were left longer than this, it was difficult to distinguish between the original viruliferous insects and their progeny.

In transferring the aphids to a test plant, the youngest fully developed leaf was inverted and held in place with a wire hook, the aphids moved to its under surface with a moistened brush, the cage attached, and the leaf released.

The indicator plants used in these experiments were *F. vesca* of the same clone as those used in the grafting experiments (1).

The experiments were all performed in the greenhouse and there the plants remained throughout the summer. In the fall the plants were set out in the field, where they remained for their second season of growth. However, during this second year, runner plants from representative individuals were allowed to root, and, when established, were returned to the greenhouse for further observation and investigation.

Separation of Yellows into Component Viruses on *Fragaria vesca*

In the experiments designed for the separation of components of yellows, the aphids were allowed to feed for a specified length of time on yellows-infected Marshall leaves and then transferred progressively through a succession of healthy plants. The acquisition feeding periods were as follows: 1½, 3, 6, 12 hr.; 1, 2, 4, 8, and 16 days. The transfer feeding periods on the test plants were likewise 1½, 3, 6, 12 hr.; 1, 2, 4, 8, and 16 days. There were three replications of each experiment, with five apids per plant.

These progressive transfers of viruliferous aphids resulted in the separation of at least two viruses that differed markedly, both in their vector relationship and in their effect on the plants. The first, designated as N in Table I, was of the nonpersistent type; the insects could transmit it immediately after their acquisition feed but lost their ability to do so in a few hours. The second, designated as P in Table I, was of the persistent type; the insects could not transmit it immediately after their acquisition feed but only after the lapse of a latent period. After this period they remained viruliferous for several days.

The Nonpersistent Component

Symptoms

The first manifestation of infection with the nonpersistent component appeared two to three weeks after inoculation. Usually one of the youngest leaves twisted, owing to the arrested development of one side of the leaf blade, and the petiole remained very short and reflexed toward the ground.

The leaves that developed in the next few weeks were all reduced in size, mottled, crinkled, twisted, and distorted, but the degree of reduction and mottling, and the degree and type of distortion, varied considerably from plant to plant.

As the disease progressed, the symptoms levelled off to a general crinkle or mottle, or, more usually, a combination of the two. Differences between individual plants were still distinct, but the newly developing leaves were less

severely affected than those on which earlier symptoms had appeared. The petioles of the old leaves flattened toward the ground, but the petioles of the leaves that were formed after the onset of the disease remained erect, though very short.

The effect on the stolons was very marked. Healthy *F. vesca* plants are prolific in stolon production, with runner plants developing at 12 to 18 in. intervals. On the diseased plants, the first effect on the stolon was a hooking back of the tip. When these hooked stolons were removed, the few subsequently produced were extremely short, the first runner plant being produced one to six inches from the parent plant, and the growth of the stolon was usually arrested after the production of one or two plants.

While reduction in leaf size, mottle, crinkle, and leaf distortion were, in a general sense, the characteristic symptoms of the nonpersistent component, the severity and dominance of individual symptoms varied considerably, not only between plants of different experiments, but also between replications of a single experiment. However, despite the variation in symptom picture, there was a tendency for the foliage of those plants with leaves only slightly reduced in size to be distinctly mottled but scarcely distorted, and the foliage of those with very small leaves to be less distinctly mottled, although in some cases small, irregular, scattered, chlorotic areas on the leaf blade caused severe leaf distortion.

During the second year, the difference in symptoms between individual plants continued. Those plants which were less severely affected, where the leaves were distinctly mottled but only slightly reduced in size, retained the same type of symptoms into their second year. Those plants in which reduction in leaf size had been extreme either died during the winter or developed into very small plants. In most of these, though the leaves were minute, specific leaf symptoms were slight or absent, but where distortion due to small scattered chlorotic areas on the leaf had occurred the first year, the same symptom again appeared.

Although most of the affected plants formed only few and abnormal stolons during the year of inoculation, normal stolon production was resumed the following spring—commensurate with the size and vigor of the individual plant.

The runner plants formed during the second season exhibited symptoms of the same type as those shown by the plants from which they were derived, and several clones with different symptom expressions were obtained. Plants from three of these clones are illustrated in Figs. 3, 4, and 5. Fig. 3 shows the mildest type of symptom. There is a distinct mottle but reduction in leaf size is slight and leaf distortion is slight or absent. Fig. 4 shows a more severe condition where there is no true mottle but small necrotic areas cause extreme leaf distortion. Fig. 5 shows another severe condition where the leaves are very small and slightly chlorotic but there is neither leaf distortion nor mottle. Fig. 2 shows a healthy runner plant of comparable age.

Transmission

The nonpersistent component was acquired by the vector after a feeding period of three hours or more, and could be transmitted within one and one-half hours after the acquisition feed (Table I). In a supplementary experiment where, after an acquisition feed of 12 hr., seven consecutive transfer feeds of an hour each were given, only one of 35 plants became infected, and the assumption must be made that an hour was not usually sufficient time for transmission.

The highest incidence of transmission was obtained in the first transfer feed, and the period during which the vector remained viruliferous was usually limited to the first two transfers. In the first one and one-half hours following the acquisition feed, there were 17 infections from 27 inoculations; in the next three hours, there were seven infections; after this period, there was only one infection (Table I).

TABLE I

INCIDENCE OF SYMPTOMS* ON *Fragaria vesca* INOCULATED BY PROGRESSIVE TRANSFERS OF APHIDS† THAT HAD FED FOR VARYING PERIODS ON YELLOWS-INFECTED MARSHALL PLANTS

Transfer	Duration of transfer feed	Duration of acquisition feed								
		1½ hr.	3 hr.	6 hr.	12 hr.	1 day	2 days	4 days	8 days	16 days
		Date of first transfer feed								
		July 7	May 18	July 7	May 18	July 7	May 18	July 7	May 18	July 7
1	1½ hr.	• • •	N • •	• N •	N • N	N N •	N N N	N N N	N N N	N N •
2	3 hr.	• • •	• • •	• • •	• N N	• • •	N N N	• • •	N • •	N • •
3	6 hr.	• • •	• • •	• • •	• • •	• • •	• • •	• • •	• N •	• • •
4	12 hr.	• • •	• • •	• • •	• • •	• • •	• • •	• • •	• • •	• • •
5	1 day	• • •	• • •	• • •	• • •	• • •	• • •	• • •	• • •	• • •
6	2 days	• • •	• • •	• • •	• • •	• • •	• • •	• • •	• • •	• • •
7	4 days	• • •	• • •	• • •	• • •	• • •	• • P	P P •	P P •	P P •
8	8 days	• • •	• • •	• • •	• • •	• • •	P • P	P • •	• P •	P • •
9	16 days	• • •	• • •	• • •	• • •	• • •	• • •	• • •	• • •	• • •

* N *denotes the symptoms of the nonpersistent virus.*

P *denotes the symptoms of the persistent virus.*

• *denotes that the Fragaria vesca plant remained healthy.*

† *These aphids were reared on British Sovereign plants. Subsequent work has shown that both the British Sovereign and the Marshall may be symptomless carriers of a virus (or viruses) similar to, if not identical with, the nonpersistent component (N) of the yellows complex. The aphids lose their ability to transmit this latent virus within six hours, and consequently would have lost it where the acquisition feed was six hours or more, therefore we believe that the possible contamination of the stock aphids with this virus does not invalidate the conclusions drawn from these experiments.*

A more serious consideration is the possible contamination of the Marshall plants used as inoculum in some of the experiments. The plants used as inoculum in July had at one time supported aphids from British Sovereign plants, and the inoculum in these tests must be considered suspect of contamination with the latent virus from British Sovereign. Since this contamination had no apparent effect on the transmission of the persistent component (P), these tests are included in Table I. However, since the possibility exists that the latent virus from the British Sovereign might have an effect on the symptom picture of the nonpersistent component, the description of this component given in the text is based solely on the plants inoculated in May, that is, those in which there was no possibility of contamination with the latent virus.

To determine whether the nonpersistent virus were quantitatively lost in the inoculation of the first two or three plants, the following experiment was performed. After an acquisition feed of six hours, aphids were divided into two groups. One group was placed on moist filter paper, and the other was transferred to healthy plants. After six hours, the insects that had been starved on the filter paper were transferred to healthy plants, and those that were already feeding were moved to fresh plants. The aphids were then allowed to feed for 12 hr. The aphids that had been starved for six hours after their acquisition feed did not transmit the virus; those that were transferred immediately transmitted it to four of five plants in their first transfer feed, but to none in their second. It appears, therefore, that it is the inability of the virus to survive in the aphid for more than six hours, rather than exhaustion of the supply of inoculum through the insects' feeding, that is responsible for limiting transmission to the first few hours after the acquisition feed.

The Persistent Component

Symptoms

The effect of the persistent component on *F. vesca* was much less severe than that of the nonpersistent. The early symptoms were confined to a few of the younger leaves and consisted of a slight cupping, slight crinkling, and a distinct mottle which was confined to the margin of the leaves, especially the tips, and which sometimes merged to give the appearance of marginal chlorosis. The older leaves remained normal in appearance and even those younger leaves with definite symptoms were little reduced in size. The petioles remained erect and there were no stolon abnormalities.

During the following summer when these plants were growing in the field, plant size was reduced and the individual leaves were smaller than normal, with leaflets slightly narrow, slightly crinkled, and slightly cupped (Figs. 6 and 7). There was seldom more than a suggestion of yellowing of the leaf margins.

When runner plants from the infected plants were moved to the greenhouse late in the summer, the marginal chlorosis appeared once again, and once more only a few of the younger leaves were affected (Fig. 8) while at the same time the plants remaining in the field continued to display only the indefinite symptoms observed earlier in the season (Fig. 9). The yellowing of the leaf margins of greenhouse plants remained in evidence for several months, but gradually disappeared and, by December, little could be found.

Transmission

The persistent component was acquired by the aphid only after feeding periods of two days or more, and required an additional four-day period within the vector before transmission was effected. The period between the acquisition

feed and the first successful transfer feed was the same regardless of the duration of the acquisition feed. After this latent period had passed, the insect remained viruliferous for at least four days (Table I).

TABLE II

INCIDENCE OF SYMPTOMS* ON *Fragaria vesca* INOCULATED BY CONTINUOUS FEEDING OF APHIDS THAT HAD FED FOR VARYING TIMES ON YELLOWS-INFECTED MARSHALL PLANTS

Duration of acquisition feed	Incidence of symptoms of nonpersistent type	Incidence of symptoms of yellows complex
1½ hr.	1/3	0/3
3 "	1/3	0/3
6 "	1/3	0/3
12 "	1/3	0/3
1 day	1/3	0/3
2 "	1/3	0/3
4 "	3/3	2/3
8 "	2/3	1/3
16 "	3/3	0/3

** Denominator indicates the number of plants inoculated. The numerator indicates the number of plants showing symptoms.*

Whereas with the nonpersistent component the period between inoculation and the appearance of symptoms was relatively short and constant, with the persistent component this period was longer and varied with environmental factors. In the experiments illustrated in Table I, the plants of alternate experiments were inoculated in May; those of intervening experiments, six weeks later. The first symptoms of the persistent component appeared in both groups at approximately the same time—the end of August—despite the six weeks' difference in time of inoculation.

TRANSMISSION OF THE YELLOWS COMPLEX TO *Fragaria vesca*

In an attempt to obtain insect transmission of the yellows complex, aphids were given varying acquisition feeds (Table II), transferred to *F. vesca* plants, and allowed to remain there for 24 days.

Symptoms of the nonpersistent component appeared in at least one plant of each acquisition feeding period. As in the progressive-transfer series, these symptoms varied in severity from plant to plant, irrespective of the length of the acquisition feed. However, in at least three plants in the four-day and eight-day acquisition feed experiments, reduction in size of the younger leaves became extreme with the passing weeks, the mottle and crinkle became less distinct, and finally death of all but the youngest leaves produced a condition indistinguishable from that produced by graft transmission of the complex

Recombination of the Nonpersistent and Persistent Components of Yellows in *Fragaria vesca*

Simultaneous inoculation of *F. vesca* plants by two groups of aphids, one carrying the nonpersistent component and the other the persistent component, has produced symptoms more severe than those caused by either component alone. In this experiment, one group of aphids was given an acquisition feed of 24 hr. on a *F. vesca* plant with the nonpersistent component and the other group an acquisition feed of four days on a *F. vesca* plant with the persistent component. Both groups were transferred to test plants on the same day. A transfer feed of three days was allowed for the nonpersistent component and of 16 days for the persistent.

The first symptom was the twisting of the youngest leaves, characteristic of of the nonpersistent component, but the leaves that were subsequently formed were much smaller than comparable leaves on plants affected with the nonpersistent component alone. These leaves showed neither the mottle or distortion of the nonpersistent, nor the cupping and yellow-edge of the persistent component, but more closely resembled the minute, relatively well formed leaves of plants inoculated with yellows by grafting (Fig. 10).

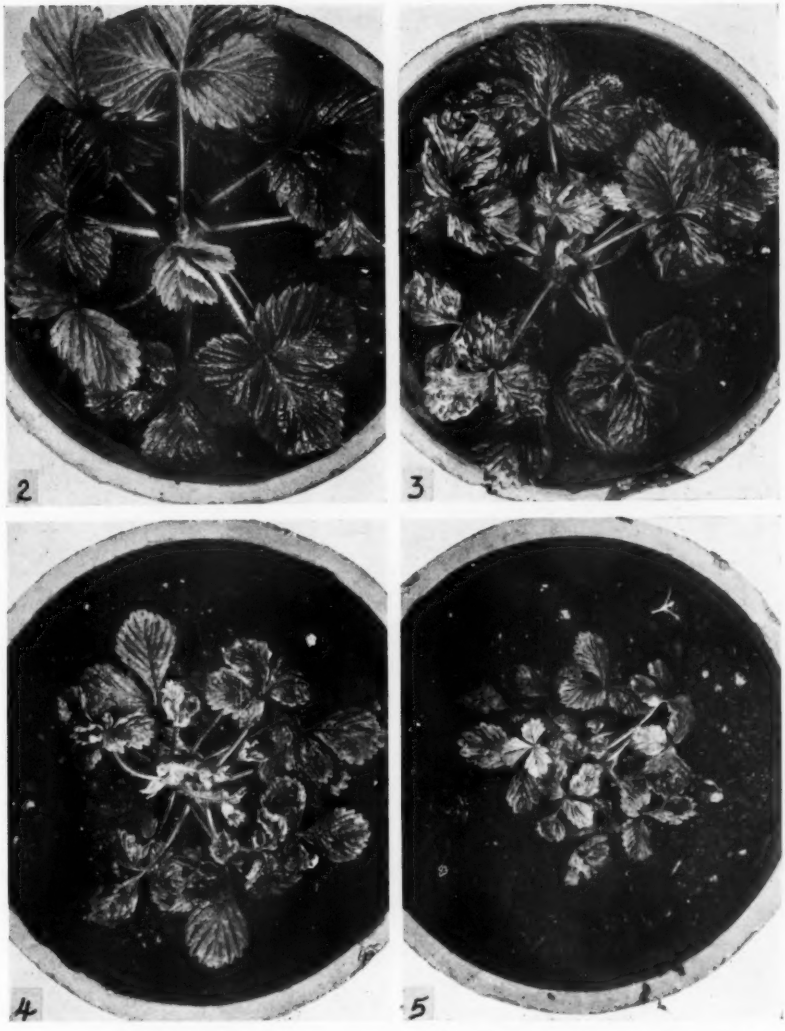
A more detailed account of the recombination of the components of yellows will be presented in a later paper.

Effect of the Components of Yellows on the British Sovereign Variety

Concurrent with the progressive transfers of viruliferous aphids through successive groups of healthy *F. vesca*, similar experiments were performed, with British Sovereign as the test plant. The methods used for inoculation were the same as those used for *F. vesca*, except that there were five instead of three replications of each experiment.

Results were less distinctive than with *F. vesca*, but on the basis of observations extending over a period of 18 months a few generalizations may be made. The nonpersistent component had no apparent effect on the British Sovereign. Plants which corresponded to those of the first two transfers in Table I were no less vigorous than the check plants. Plants which corresponded to those of later transfers remained normal in appearance during the season of inoculation, but, during their second year, many began to degenerate. Symptoms were not definite, but the plants were much smaller than normal, the leaves smaller than normal, and the petioles tended to flatten toward the ground, giving the appearance of British Sovereign plants grafted to yellows-infected Marshall (1).

In the experiments with *F. vesca*, symptoms of the persistent component were confined to plants in the seventh and eighth transfer of those experiments where the acquisition feeding period had been two days or more (Table I). In the experiments with British Sovereign, symptoms of degeneration were



Runner plants of *Fragaria vesca* from clones showing different types of symptoms of the nonpersistent component of yellows. (Photographed in November when the plants were approximately five months old.)

FIG. 2. Normal plant.

FIG. 3. Mild type with distinct mottle but little reduction in size and no leaf distortion.

FIG. 4. More severe type where small, scattered, necrotic areas on the leaf blade cause severe distortion.

FIG. 5. Severe type with marked reduction in leaf- and plant- size, but no mottle or leaf distortion.

Symptoms of the persistent component of yellows on *Fragaria vesca*.

FIG. 6. Normal plant—check for FIG. 7. (Photographed in July.)

FIG. 7. Plant growing in the field the year following inoculation, showing reduced size and slight leaf cupping. (Photographed in July.)

FIG. 8. Runner plant, showing the mottling and crinkling which appeared on the younger leaves after the plants were returned to the greenhouse. (Photographed in September.)

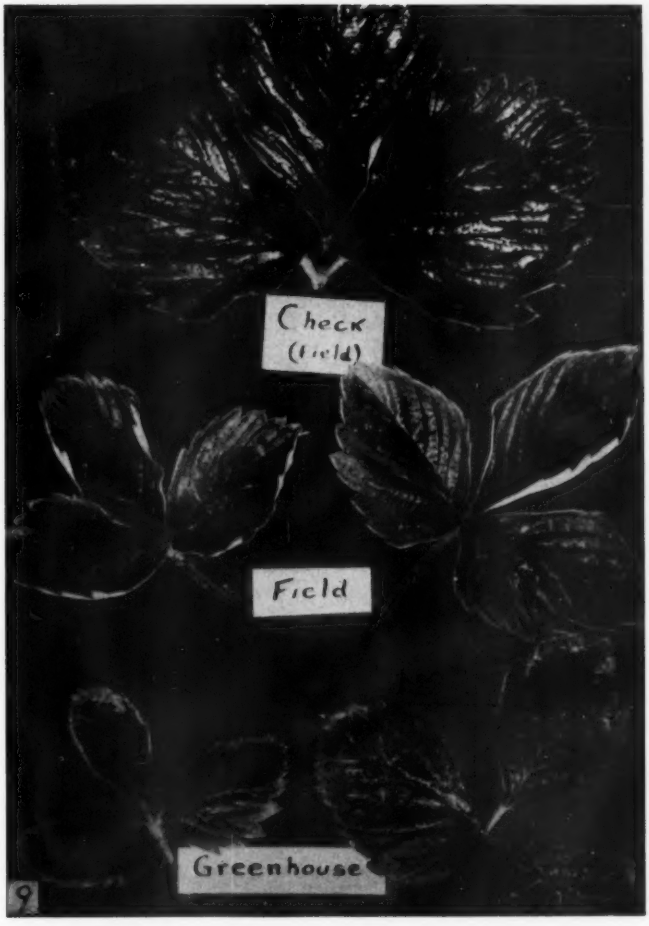


FIG. 9. Leaf detail of *Fragaria vesca* with the persistent component of yellows, showing the variation in symptoms between field and greenhouse plants. (Photographed in September.)

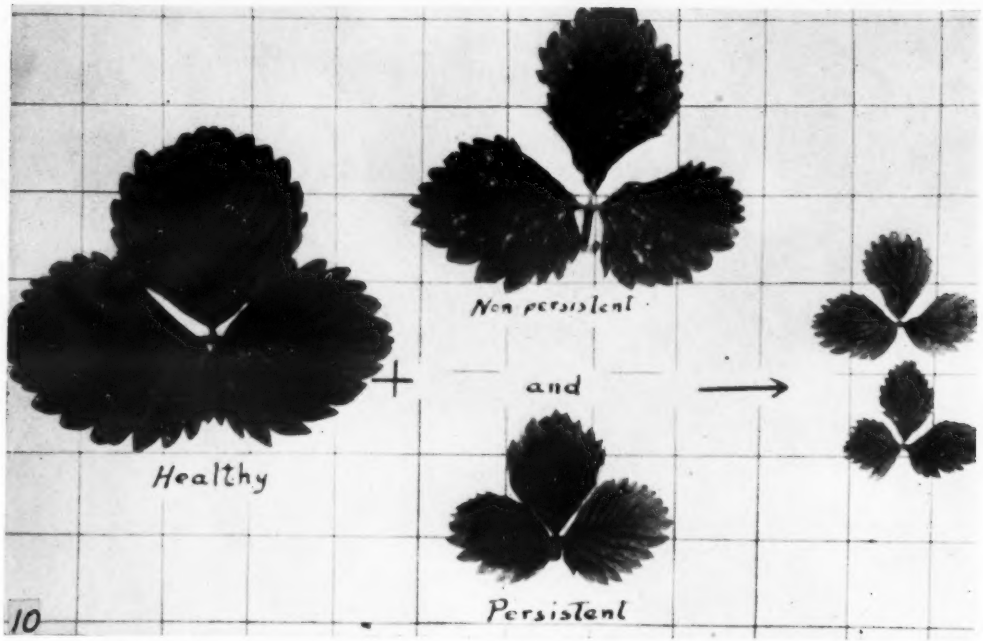


FIG. 10. Effect of combination of nonpersistent and persistent components of yellows on *Fragaria vesca*.

not so confined, but the greatest number of affected plants occurred in transfers five, six, and seven and in the experiment where the acquisition feed had been eight days.

Discussion

The isolation of component viruses from yellows-infected Marshall plants and their subsequent recombination to produce yellows in *F. vesca* have shown that the yellows disease of the Pacific Northwest and the yellow-edge disease of England are etiologically analogous if not identical.

In their work in England, Prentice and Harris (2, 3, and 4) describe three viruses isolated from complexes occurring in strawberry plants. These viruses they designate as viruses 1, 2, and 3. Viruses 1 and 2 were isolated from a yellow-edge diseased plant; virus 3, together with virus 1, was isolated from a plant with severe crinkle.

Virus 1 produced chlorotic spotting and leaf distortion in *F. vesca*. The vector required an acquisition feed of at least one hour and the incidence of transmission increased with longer feeds. There was no latent period and the vector's ability to transmit the disease was limited to about three hours. Transfer feeds as short as 10 min. sufficed to transmit the disease. The first symptoms appeared in two to three weeks.

The properties of the nonpersistent component isolated from the yellows-infected Marshall clone in our experiments were similar to those of Prentice and Harris' virus I. The vector relationship was the same, both with respect to the requirements of the acquisition feed and to the lack of a latent period. However, unlike Prentice and Harris' virus 1, transmission was seldom effected with transfer feeds of less than one and one-half hours.

Prentice and Harris report considerable variation in symptom severity with their virus 1. They suggest that this variation might be caused either by individual plant reaction or by the existence of two or more strains of one virus. A similar variation in symptom expression occurred with the nonpersistent virus in our experiments. However, vegetative propagation of several plants with different symptom expressions demonstrated that each runner plant displayed symptoms similar to those of the parent plant from which it arose. It is apparent, therefore, that the variation in symptoms is not due to individual plant reaction. Of the three types, the symptoms of one (Fig. 4) correspond very closely to the severe symptoms of virus 1 illustrated by Prentice and Harris (4). The other two types (Figs. 3 and 5) were unlike any component illustrated by Prentice and Harris; the mild symptoms of virus 1 which they illustrate were not obtained from the source of yellows used in these experiments.

Virus 2 of Prentice (3) produced slight chlorotic spotting, slight chlorosis, and leaf cupping in *F. vesca*. The vector required an acquisition feed of at least 24 hr. and there was evidence of a latent period, although its duration was not clearly defined. The vector retained its ability to transmit the disease for several days. The first symptoms appeared in four to eight weeks.

The persistent component isolated from the yellows-infected Marshall clone was similar in vector relationship and in incubation period within the plant to virus 2 of Prentice (3), but the symptoms it produced in *F. vesca* differed from those produced by virus 2 in that there was no general chlorotic flecking on the smaller veins of the young leaves. The symptoms most closely resembled those described by Prentice for the persistent virus he isolated from Huxley's Giant, a virus that he suspected to be a mild strain of virus 2.

Virus 3, isolated by Prentice (4) from plants with severe crinkle, and recombined with either virus 2 or viruses 1 and 2 to give severe yellow-edge, was not obtained from the source of yellows used in our experiments.

The nonpersistent component produced no visible symptoms on British Sovereign. The persistent component, while it caused no characteristic symptoms, did result in severe degeneration of many plants. This British Sovereign stock was later found to be carrying a latent virus of the nonpersistent type. It is possible that the degeneration observed in the plants inoculated with the persistent component was due to the combined effect of this latent virus and the persistent component introduced from the yellows complex. Determination of the effect on British Sovereign of the persistent component alone will not be possible until a virus-free stock of this variety is established.

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THE ANNUAL CYCLE OF SIZE CHANGES IN THE FUSIFORM CAMBIAL CELLS OF *CHAMAECYPARIS* AND *THUJA*[1]

By M. W. Bannan[2]

Abstract

In stems exceeding a few inches in diameter most of the pseudotransverse divisions involved in the multiplication of fusiform cambial cells occur toward the end of the growing season. Often these aestival transverse divisions are immediately followed by extensive elongation of the new-formed cambial cells, especially at their overlapping tips. In the succeeding year relatively slight elongation ensues during the development of the first quarter of the annual ring, but through the succeeding quarters the amount of extension increases and is usually maximal in the final quarter. The actual rates of elongation remain undetermined. The multiplication of fusiform initials is accompanied by loss, most of the failure taking place during the last quarter. Generally the fusiform initials with the most extensive ray contacts survive and enlarge, and those with poor ray associations fail or are reduced to potential ray initials. The elongation and multiplication of fusiform initials tend to produce local ray deficiencies. Reduction of the fusiform initials with the poorest ray contacts to ray initials rectifies to varying extent the ray shortages in those areas.

Introduction

In conifers the multiplication of fusiform initials in the tangentially expanding cambium results almost entirely from pseudotransverse division. The frequency of these divisions has been dealt with in two previous publications (Bannan (1) and Whalley (10)). Later articles described the elongation of fusiform initials following pseudotransverse division (Bannan and Whalley (3)) and the transformations accompanying loss of cells from the cambium (Bannan (2)). The present paper submits the results of investigations on the multiplication, enlargement, and reduction of cambial cells in relation to annual growth.

Materials and Methods

Since both the secondary xylem and secondary phloem replicate the basic cell pattern in the cambium, either of these tissue systems may be utilized for ascertaining the history of change in that meristem. However, for the determination of the nature and rate of alteration in relation to annual increment the xylem was used in preference to the phloem because of the greater ease in recognizing the boundaries of yearly growth in the former. Trees of relatively small size, D.B.H. mostly 6–10 in., were selected and serial tangential sections cut from the outer annual rings. The sections were examined and the locations of pseudotransverse divisions leading to the multiplication of fusiform cambial cells noted. Those particular developmental series consequent to pseudotransverse division during the last portion of each year's growth were selected for the recording of data. The history of the resulting sister fusiform initials

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[2] *Assistant Professor in Botany.*

was traced through the growth of the succeeding year, and data acquired on the increase in length, the occurrence of subsequent anticlinal divisions, and the time of loss of derived cambial cells when this transpired. The studies were based on eastern and western North American representatives of *Chamaecyparis* and *Thuja*, namely *C. thyoides* (L.) BSP., *C. Lawsoniana* (Murr.) Parl., *T. occidentalis* L., and *T. plicata* D. Don. A total of nearly 1100 developmental series was studied.

The Length of Transversely Dividing Fusiform Initials

In the type of material studied most of the transverse divisions involved in the multiplication of fusiform initials occur toward the end of the growing period when the last portion of the late wood is being produced. Data on length of the fusiform initials undergoing pseudotransverse division at this time are found in Table I. Here is shown the length of the dividing cells expressed in the form of the mean and the standard deviation. In this connection it should be noted that the data were of necessity obtained from the derived elements in the secondary xylem, and hence are in error to the extent of the elongation of the tracheids during maturation. Differences in the mean are apparent among the species investigated. The transversely dividing cells tended to be longer in *Chamaecyparis* than in *Thuja*, and in each genus the length was slightly greater in the eastern than in the western representatives. The extent of variation relative to the mean was of similar magnitude in the different species. The standard deviation was highest at 0.40 mm., in *Chamaecyparis* where the cells were longer. The coefficient of variation (the standard deviation in relation to the mean) fell within the limits 12 to 14% in the four species.

It may be concluded from the data in Table I that size of the fusiform initials is an important element in their transverse division. Length alone, however, does not appear to be the only factor involved. Occasionally a second pseudotransverse division follows soon after the first, one of the sister (daughter) initials being transversely divided before much elongation has accrued. Fig. 1 illustrates such a sequence, Figs. 1*a* and 1*b* being drawn from consecutive sections. It is also noteworthy that vernal pseudotransverse divisions, which are of sporadic occurrence, usually take place in cells somewhat shorter than those transversely halved near the end of the preceding year's growth.

The data in Table I relate to the transverse division of expanding fusiform initials, one or both derived sister initials surviving and enlarging in turn. Often transverse subdivision occurs under circumstances of quite different nature. Loss of fusiform initials from the cambium happens frequently, and most of the failing cambial cells are reduced by contraction in length and tangential width, accompanied by transverse subdivision to the first or further orders. Here the anticlinal divisions occur not in expanding cells but rather in cells which seem to lag in competition with their neighbors and are suffering

TABLE I

GENERAL DATA PERTAINING TO AESTIVAL PSEUDOTRANSVERSE DIVISIONS INVOLVED IN THE MULTIPLICATION OF FUSIFORM INITIALS IN THE CAMBIUM

Species	Tree No.	Diam. in inches	Length cells at pseudotrans. div. in mm.	Mean length of anticlinal wall in mm.	No. of developmental series studied	No. series with all cambial cells living through following year
C. thyoides	1	7	3.24 ± .32	.56	21	14
	2	6	3.50 ± .48	.56	46	18
	3	8	3.68 ± .48	.51	33	15
	4	8	3.56 ± .53	.46	32	11
	5	10	3.57 ± .39	.60	59	17
	6	10	3.27 ± .39	.50	44	8
	7	9	3.38 ± .37	.51	60	9
	8	7	3.98 ± .38	.48	25	17
	Av. or total	8	3.52 ± .42	.52	320	109
C. Lawsoniana	10	8	2.91 ± .29	.48	16	8
	11	8	2.92 ± .43	.48	36	13
	12	9	3.10 ± .32	.55	36	16
	13	14	3.20 ± .49	.48	35	15
	14	15	2.94 ± .30	.63	25	2
	Av. or total	11	3.01 ± .37	.52	148	54
T. occidentalis	16	6	2.22 ± .29	.35	79	38
	17	7	2.58 ± .30	.44	53	31
	18	6.5	2.35 ± .38	.38	67	28
	19	9	2.64 ± .31	.46	28	17
	20	6	2.14 ± .27	.29	34	14
	21	7.5	2.56 ± .29	.41	58	24
	22	5.5	2.61 ± .32	.38	50	23
	23	7	2.96 ± .29	.44	46	18
	24	7	2.78 ± .31	.47	44	23
	Av. or total	7	2.54 ± .31	.40	459	216
T. plicata	26	9	2.25 ± .29	.42	11	1
	27	8	2.16 ± .35	.33	37	12
	28	9	2.55 ± .34	.47	40	8
	29	10.5	1.82 ± .27	.35	61	10
	Av. or total	9	2.20 ± .31	.39	149	31

impairment to their generative capacity. In these cells it would appear that the stimulus to transverse division originated in altered physiological conditions and the size element, especially length, was relatively unimportant.

Trends in Elongation of Fusiform Cambial Cells

Before dealing with the trends in enlargement and reduction of fusiform initials it is pertinent to recall certain references to this topic in a previous publication (Bannan and Whalley (3)). In that article it was pointed out

that the rate and amount of longitudinal extension varies widely among neighboring cambial cells. While some enlarge at diverse rates others contract and lapse into maturation or are reduced to smaller units some destined to become ray initials. In the case of the expanding initials elongation may proceed in a steady manner or it may be fitful, being influenced apparently both by internal circumstances within each cell and by the local environment. Sometimes marked differences in extent or rate of elongation develop at opposite ends of the same fusiform initial. The existence of this very great individual fluctuation must not be overlooked when reference is made to summarized tabulated data which show only the mean behavior. It should be emphasized that in few if any individual cells does growth conform to the smoothed curve of the mean.

With few exceptions the multiplication of fusiform cambial cells is effected by pseudotransverse division, usually near the center of the dividing cell, but the pitch and length of the oblique anticlinal wall fluctuates widely. When first observed in the derived xylem elements the length of the partitioning wall ranges from short and almost transverse (Fig. 2) to more than one-third the length of the mother cell (Fig. 3). As shown in Table I the mean length was 0.52 mm. in *Chamaecyparis thyoides* and *C. Lawsoniana*, 0.40 mm. in *Thuja occidentalis* and 0.39 mm. in *T. plicata*. These mean values are approximately one-fifth to one-seventh the length of the cells undergoing transverse division.

Exact length of the partitioning anticlinal wall at the beginning is of course not determinable from the derived xylem elements because of slight but varied elongation of the latter during maturation, and further to the fact that in many cases the sister fusiform initials apparently undergo some elongation prior to the production of the first permanent derivatives. Evidence of elongation immediately following cytokinesis is to be found in the shape and position of the overlapping tips of sister cells, both in relation to each other and to neighboring cells. Some examples are illustrated in Figs. 1, 4, and 7. Figs. 1*a* and 1*b*, 4*a* and 4*b*, 7*b* and 7*c* were drawn in each case from consecutive sections and they show the size and shape of the xylem cells just before and after pseudotransverse division. For instance, Fig. 4*a* depicts the last tracheid produced before pseudotransverse division and Fig. 4*b* the first xylem derivatives of the new-formed sister cambial cells. The configuration of the overlapping tips in Fig. 4*b*, especially in relation to the lower adjoining ray, testifies to extensive early elongation of the new-formed sister cells along the

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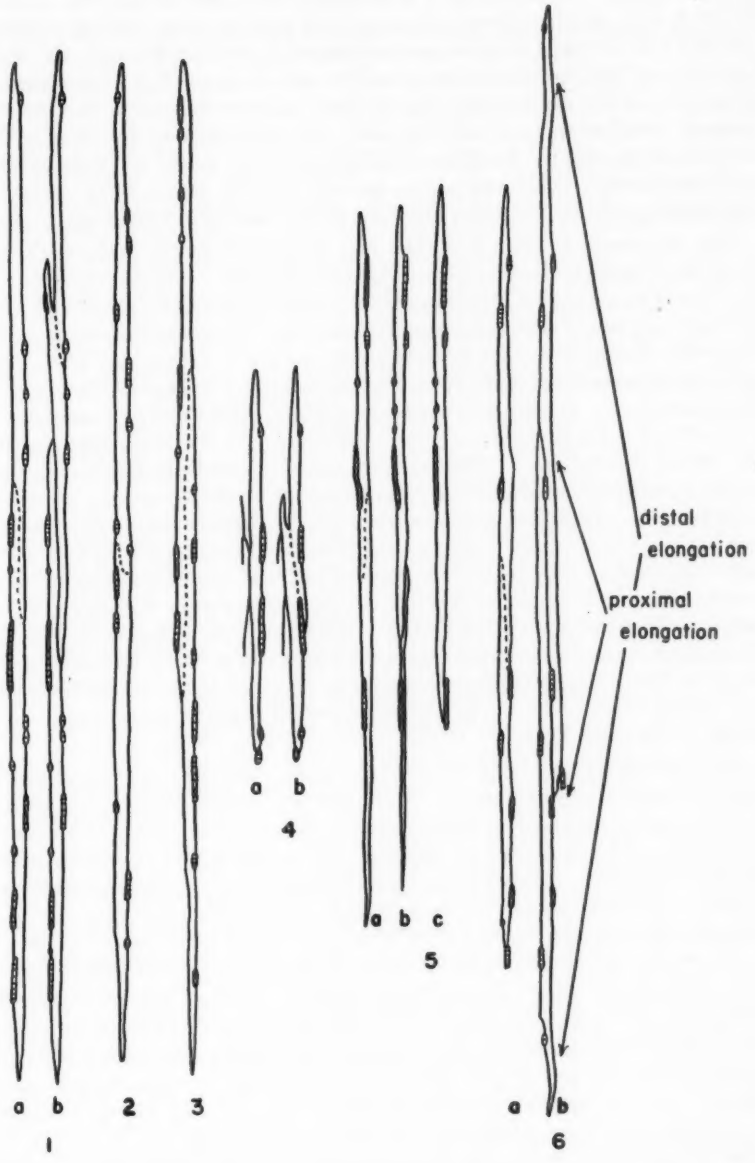
FIGS. 1 – 6. Drawings from tangential sections of the outer wood of stems which are to be interpreted as showing the nature of the pseudotransverse divisions involved in the multiplication of fusiform cambial cells. FIG. 1. *Chamaecyparis thyoides*, showing aestival pseudotransverse division soon followed by division of the upper sister initial. FIGS. 2 and 3. *C. thyoides*, range in length of the partitioning anticlinal wall as first observed in the derived xylem elements. FIG. 4. *Thuja occidentalis*, drawings from consecutive sections showing extensive elongation of overlapping tips of sister cells after pseudotransverse division. FIG. 5. *T. occidentalis*, drawings from late wood, and at the midway point and at the end of the following year's growth, showing loss of the lower sister cambial cell and downward elongation of the upper sister. FIG. 6. *C. thyoides*, showing elongation of sister fusiform initials after pseudotransverse division.


a
b
1
2
3
a
b
4
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c
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6
distal
elongation
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elongation

axis of overlap. Again in Fig. 7 it is evident that pseudotransverse division of cell X was quickly followed by an upward growth of the derived daughter cambial cell X^{II} (Fig. 7*c*), the great increase in overlap between the sister cells X^{I} and X^{II} coinciding with loss of the adjoining cell Y. The amount of elongation of the overlapping tips of sister fusiform initials in the interval between pseudotransverse division and the production of the first xylem derivatives apparently fluctuates considerably. No doubt the elapsed time is of considerable importance. Also, as shown in Fig. 7*c*, the behavior of the surrounding cells has a decided influence on the amount of elongation.

The increase in overlap from the first to second xylem derivatives laid down after pseudotransverse division also varies widely. Among other factors the time of year seems highly important. As a rule elongation is greater after aestival than vernal pseudotransverse divisions. Of course, it must be recognized that the successive tracheids in any radial file of cells are not necessarily consecutive derivatives of the fusiform initials in the uniseriate initiating layer of the cambium. During the development of the early wood especially extensive multiplication of incipient xylem cells occurs in the xylemward portion of a broad cambial zone. The exact sequence of periclinal divisions in such series of multiplying cells can only be deduced after careful study. Probably these secondary periclinal divisions occur more frequently during the development of early wood than of late wood. For the present, let it suffice to say that elongation as apparent in the successive xylem derivatives tended to be greater after aestival than after vernal pseudotransverse divisions. When the transverse anticlinal division took place at the end of the growing season the increase in overlap from the last late-wood elements to the first early-wood tracheids in the succeeding ring ranged from $-75\ \mu$ to $320\ \mu$ in *Thuja occidentalis*, with a mean of $37\ \mu$. In *Chamaecyparis thyoides* the range was $-55\ \mu$ to $640\ \mu$, the mean $88\ \mu$.

The early elongation along the overlap was usually followed by some slackening in the amount of extension. Data on the changing trends in elongation through the growth of a year after aestival pseudotransverse division are given in Table II. The data show the total increase in length of the sister initials at the two remote tips, referred to as distal elongation (see Fig. 6), and along the overlap, described as proximal elongation. Obviously the data in each instance relate to two sites of elongation. The data show the additions to length at the end of successive quarters of the year's growth, the divisions being on a spatial rather than a temporal basis. Time relationships are unknown and probably varied in the different specimens studied.

In both *Chamaecyparis* and *Thuja* the proximal elongation of sister fusiform initials during the first quarter of the year's growth tended to be relatively limited. On the average it was only approximately twice the increase recorded between the production of the first and second xylem elements laid down after pseudotransverse division. It seems probable that the elongation during the first quarter was little more than the actual increase prior to and immediately following the first xylemward periclinal division of the sister initials after origin

TABLE II

THE DISTAL AND PROXIMAL ELONGATION OF SISTER FUSIFORM CAMBIAL CELLS DURING THE YEAR FOLLOWING ORIGIN IN AESTIVAL PSEUDOTRANSVERSE DIVISION

Species	Tree No.	Annual increment								Total elongation in μ	Ring width in mm.
		First quarter		Second quarter		Third quarter		Fourth quarter			
		Dist.	Prox.	Dist.	Prox.	Dist.	Prox.	Dist.	Prox.		
C. thyoides	1	114	96	67	57	182	153	248	126	1043	2.50
	2	119	119	86	124	195	122	400	252	1417	1.51
	3	220	196	239	193	235	160	328	227	1798	2.19
	4	150	76	141	138	186	217	423	157	1488	1.46
	5	232	255	196	324	227	215	330	267	2046	1.94
	6	242	116	290	227	201	146	176	154	1552	1.54
	7	172	90	191	222	333	256	417	417	2098	2.65
	8	188	90	251	256	311	208	220	177	1701	1.87
	Av.	180	130	183	193	234	185	318	222	1643	1.96
C. Lawsoniana	10	56	41	79	13	268	253	347	185	1242	1.23
	11	263	210	224	168	312	244	525	342	2288	2.38
	12	−33	41	53	69	155	181	293	347	1106	0.98
	13	94	129	147	63	219	228	310	410	1600	1.19
	Av.	95	105	125	78	238	227	369	321	1559	1.45
T. occidentalis	16	45	60	98	104	183	200	137	126	953	1.78
	17	65	81	72	78	150	143	426	307	1322	1.90
	18	57	64	123	154	210	211	218	174	1211	2.90
	19	44	37	64	77	180	129	130	139	800	1.05
	20	139	118	189	164	154	152	117	61	1094	2.63
	21	73	51	99	87	174	137	215	105	941	1.31
	22	75	85	146	190	297	210	256	210	1469	2.90
	23	94	67	85	113	109	129	194	171	962	0.96
	24	11	16	59	72	121	136	407	249	1071	2.95
	Av.	67	64	104	115	175	161	233	171	1092	2.04
T. plicata	27	50	92	165	209	209	117	112	45	1059	1.55
	28	86	76	106	67	134	128	264	114	975	1.50
	29	81	51	80	62	196	59	114	69	712	1.85
	Av.	72	73	117	113	180	121	163	76	915	1.63

in pseudotransverse division. Perusal of the data in Table II reveals some noteworthy differences between samples. For instance, among the specimens of *Thuja occidentalis* with the widest annual rings, the average increase in the overlap of sister fusiform initials during the first quarter ranged from 16 μ in tree No. 24 to 118 μ in No. 20.

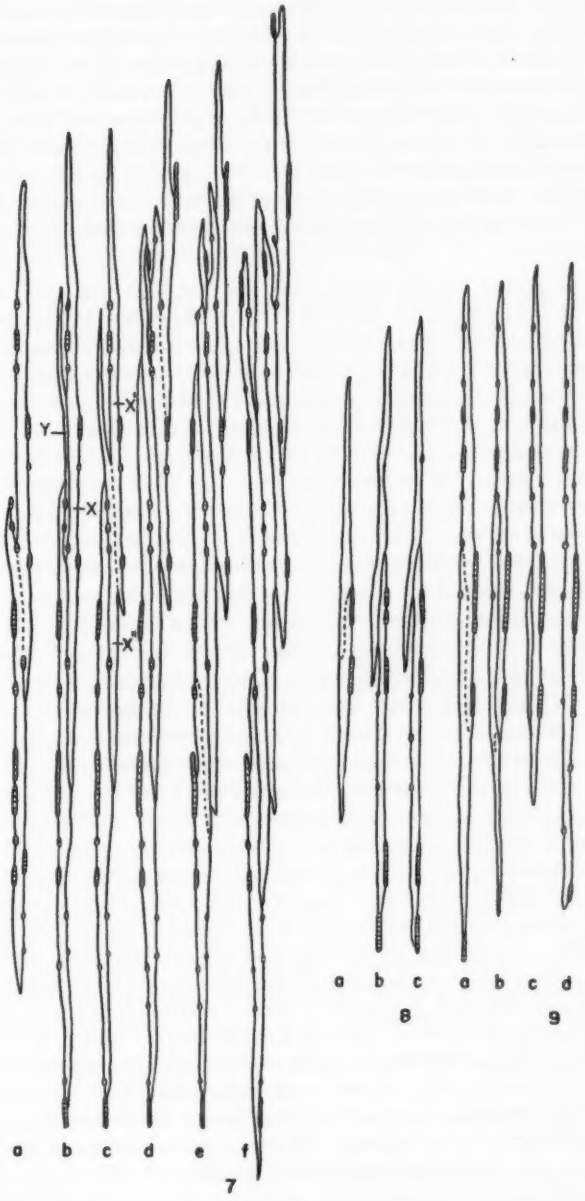
Amount of distal extension, at the remote ends of sister fusiform initials, likewise varied widely. In *Thuja occidentalis* average elongation during the first quarter fluctuated from 11 μ in tree No. 24 to 139 μ in tree No. 20. Even wider variation was noted in *Chamaecyparis Lawsoniana*. In tree No. 12

there was a small net loss whereas in tree No. 11 the distal extension was the greatest in any of the specimens studied. A comparison of distal and proximal elongation during the first quarter shows these to have been more or less similar in extent in both species of *Thuja* and in *Chamaecyparis Lawsoniana*, whereas in *C. thyoides* elongation of the two distal tips surpassed that along the overlap.

Elongation tended to increase in amount through the second and third quarters with a maximum usually being attained in the last quarter. Some differences were noted in the various specimens, as for instance in *Thuja occidentalis* tree No. 20 where the greatest elongation took place in the second quarter and the least in the final quarter. The aspect of the annual rings in this specimen differed from that in most, the late wood comprising nearly one-half the annual rings. A decided contrast is afforded in *Chaemaecyparis Lawsoniana* tree No. 12 where only slight elongation occurred in the first quarter while that in the fourth quarter was very extensive. On the whole 37% of the combined proximal and distal elongation took place in the final quarter in *Thuja occidentalis*, 33% in *Chamaecyparis thyoides* and 45% in *C. Lawsoniana*. It may be noted that during the last quarter distal growth tended to exceed that of the overlapping tips.

With regard to the data in Table II it should be emphasized that these do not show the total elongation of all cambial cells during the growth of one year. The values presented are the mean extension at the distal extremities and along the axis of the first partitioning anticlinal wall (the overlap) in lineal series where all derived cambial cells endured to the end of the growing season. For the most part the data were derived from those particular lineal series where aestival pseudotransverse division was followed in the succeeding year simply by expansion of the two sister cambial cells, no further multiplication of fusiform initials occurring. However, in some cases one or both sister cambial cells were in turn subdivided within a year, with the result that further extension took place along the new-formed overlap resulting from the second transverse division. This additional elongation has not been included in Table II because of the obvious difficulty in maintaining the distinction between proximal and distal elongation in such a series of multiplying cells. At times this additional extension was considerable. For instance, in tree No. 13, *Chamaecyparis Lawsoniana*, the second pseudotransverse division was occasionally followed by a third. Fig. 7 illustrates such a sequence of pseudotransverse divisions, the first (Fig. 7*a*) occurring toward the end of a year's growth, a second (Fig. 7*c*) during the development of the first part of the late wood in the succeeding year, and the third division (Fig. 7*d*) slightly later.

FIGS. 7–9. FIG. 7. *Chamaecyparis Lawsoniana*, multiplication of fusiform initials by successive pseudotransverse divisions during interval of one year, from summer to summer. FIG. 8. *Thuja occidentalis*, stages during span of one year showing survival of sister fusiform initial poor in ray contacts, an unusual happening. FIG. 9. *C. thyoides*, stages in growth of a year showing loss of sister cambial cell poor in ray contacts, a common occurrence.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7
a b c a b c d
8 9

The time lapse between the stages shown in Figs. 7*a* and 7*f* is accordingly one year. During that interval three successive pseudotransverse divisions occurred in the lineal series in question yielding a total of five fusiform initials all of which survived. Such complete survival is unusual in rapidly expanding series. Generally a high proportion of the derived cambial cells disappears. Combined length of the five initials at the stage in Fig. 7*f* was 11.2 mm. as compared with a length of 3.7 mm. for the progenitor in Fig. 7*a*. Hence in this particular lineal series a threefold extension occurred during the span of one year. The overlapping arrangement of the five derived cells is particularly noteworthy.

Attention should also be directed to the fact that the data in Table II pertain only to elongation in those expanding series where both sister fusiform initials, or all their daughter cambial cells if further multiplication ensued, survived through the succeeding year's growth. Generally some loss occurred during the year. This was the case in 53% of the lineal series studied in *Thuja occidentalis*, in 63% in *Chamaecyparis Lawsoniana*, and in 66% in *C. thyoides*. As shown in the final two columns in Table I the proportion fluctuated widely in the various samplings. In *Chamaecyparis thyoides* tree No. 8 both sister cambial cells lived through the year following origin in 17 of the 25 series studied, whereas in tree No. 7 the proportion was 9 in 60. After such loss the surviving sister cambial cell usually experienced a sudden elongation, the proximal tip quickly extending into the vacated space. Fig. 5 illustrates the steps in such development. After aestival pseudotransverse division (Fig. 5*a*) the lower sister cambial cell "shrank" (Fig. 5*b*) and was lost from the cambium, allowing a sudden downward extension of the upper sister (Fig. 5*c*). Owing largely to these abrupt extensions the average annual elongation of the surviving sisters in series where loss occurred tended to surpass that in lineal series where all fusiform initials persisted. In the various samplings the increase in elongation ranged from 4 to 53% and the mean was 26%. Owing to the sporadic multiplication of fusiform cambial cells at various times through the growing season, and more especially to the high frequency of loss, it is very difficult to arrive at a satisfactory estimate of the mean annual elongation of fusiform initials. The data in Table II have shortcomings since they relate only to the more stable development series. However, they are useful for comparison of samples and species and they together with information from other sources provide the basis for the following conclusions.

1. Considerable variation in cambial behavior relative to girth increase exists among samplings from trees of similar size and growth rate. Sometimes dissimilarities also occur in different sectors around the circumference and in the growth of successive years in the same tangential locus. Because of such variability the determination of the true means in different specimens or in different species requires very extensive and laborious investigation.

2. Although much fluctuation occurs there is a general tendency for the yearly elongation of fusiform initials to be greater in trees with the wider annual rings. This shows up in *Thuja occidentalis* when trees numbered 17, 18,

20, and 22 on the one hand are contrasted with numbers 19, 21, and 23. 3. With some exceptions the amount of elongation tends to increase during the deposition of the third and more especially the fourth quarters of the annual ring. 4. In trees of similar size and growth rate the annual elongation of fusiform initials is greater in *Chamaecyparis* than in *Thuja*. This applies both to the absolute elongation and to growth in relation to the length of the cambial cells. 5. In general the cambium in *Chamaecyparis Lawsoniana* and *C. thyoides* appears to be less stable than in *Thuja occidentalis*. More fusiform initials are lost from the cambium, the remainder elongate faster, and the pattern of cells in the cambium changes more rapidly and to a greater extent.

The Time of Loss of Fusiform Cambial Cells

As mentioned earlier when the size of transversely dividing fusiform initials was being discussed, pseudotransverse division takes place under varied circumstances. It is usually the means by which multiplication of fusiform cambial cells is effected, elongating initials dividing to produce daughter initials which in turn repeat the cycle of elongation and division. In other cases the transverse divisions occur not in elongating cells but rather in fusiform initials which have either begun to or are about to become reduced in size preparatory to loss from the cambium. All data on pseudotransverse divisions in the present paper refer to divisions in developmental series of the former type, that is, expanding series in which aestival pseudotransverse division was followed by enlargement of one or both resulting sister cambial cells. Even among these series, however, loss was of frequent occurrence, the proportion of series in which loss transpired in the year's growth following aestival pseudotransverse division ranging from 32 to 85% in the various samplings. Often one of the sister fusiform initials resulting from the aestival pseudotransverse division lapsed into maturation or underwent reduction, or, if subsequent transverse divisions ensued, one or more of the derived cambial cells disappeared. The transformations associated with loss have been described elsewhere (Bannan (2)). In review it may be stated that the majority of the vanishing fusiform initials are subdivided by one or a succession of more or less transverse divisions. The resulting segments shorten through the succeeding periclinal divisions, some disappearing and others persisting to become ray initials. Other fusiform initials undergo size reduction during the final periclinal divisions immediately prior to loss of generative capacity and apparently maturate to more or less abnormal permanent elements.

Although some fluctuation was discovered in different samples there was a definite trend with regard to the time of loss of fusiform initials. As shown in Table III the losses tended to increase during the development of the successive quarters of the year's growth. In the aggregate more than 40% of the loss occurred during the final quarter.It is noteworthy that the most extensive elongation of the surviving fusiform initials took place at this time. The coincidence is highly significant.

TABLE III

TIME OF LOSS OF FUSIFORM CAMBIAL CELLS IN RELATION TO THE ANNUAL INCREMENT

Species	No. of observations	Per cent of loss			
		First quarter	Second quarter	Third quarter	Final quarter
C. thyoides	276	14	27	29	30
C. Lawsoniana	122	17	12	13	58
T. occidentalis	295	9.5	17	30.5	43
T. plicata	172	10	18	33	39
Av.		12.5	18.5	26.5	42.5

The Ray Contacts of Fusiform Cambial Cells

One of the most notable features of cambial activity in relation to girth increase is the diversity in behavior of neighboring fusiform initials. While some undergo reduction in size or subdivision to ray initials others enlarge, the elongation of adjoining cells proceeding at different rates and to diverse extents. The differences in behavior apparently stem from internal conditions within each cell, these in turn being influenced to varying degree by the nature of the local environment. The latter varies both in the size, type, and number of cells which constitute the immediate vicinity of every fusiform initial, and apparently in the physiological conditions which obtain in each of these cells. One of the most obvious of the physical variables is the extent of contact between fusiform initials and the cambial portions of the vascular rays. Some fusiform initials touch numerous rays, others few or rarely none.

With the object of determining whether relationship existed between cambial cell behavior and extent of ray contact, studies were made in *Chamaecyparis thyoides* of contrasting sister cambial cells. One hundred and twenty-nine cases were selected in which aestival pseudotransverse division was followed by loss of one sister during the succeeding year and survival and enlargement of the other sister. Length of the sister initials just after their origin in pseudotransverse division and the size and number of the adjoining rays were determined from the derived xylem elements. The data revealed that the sister cambial cells destined to survive were at the beginning approximately 2090 μ long and had contacts with an average of 6.1 rays made up of a combined total of 21.2 rows of cells. By way of contrast the sister cambial cells which were shortly to fail were approximately 1940 μ long and touched 4.9 rays with a total mean height of 16.1 cells. The obvious deduction from these observations is that sister cambial cells which are larger and, more importantly, have the best ray contacts are most apt to endure.

Additional data on the ray contacts of fusiform cambial cells are provided in Table IV. Again the data pertain to lineal series beginning with aestival pseudotransverse division, but here the data were derived not only from lineal

TABLE IV

THE RAY CONTACTS OF FUSIFORM CAMBIAL CELLS WHICH CONTINUE TO ELONGATE AND OF THOSE WHICH FAIL DURING THE YEAR FOLLOWING ORIGIN IN PSEUDOTRANSVERSE DIVISION

Species	Tree No.	Total height of contact rays in cells		Number of contact rays		No. of cells in contact rays per mm. of length of cambial cell	
		Living fusif. initial	Failing fusif. initial	Living fusif. initial	Failing fusif. initial	Living fusif. initial	Failing fusif. initial
C. thyoides	1	16.8	9.1	7.1	4.1	7.7	5.2
	2	26.1	15.3	7.4	4.1	11.0	8.5
	3	25.4	19.5	6.9	5.3	10.0	9.4
	4	24.9	15.4	6.1	4.0	11.1	8.4
	5	25.4	15.9	6.7	4.8	10.2	7.8
	6	21.1	14.6	6.8	4.5	8.7	7.8
	7	31.4	17.9	6.4	3.8	13.3	9.2
	8	24.3	15.9	8.0	5.3	8.7	8.0
	Av.	24.5	15.4	6.9	4.5	9.8	7.8
C. Lawsoniana	10	21.5	12.4	5.3	3.9	10.7	7.8
	11	22.8	14.8	5.0	3.4	10.5	8.0
	12	20.1	14.4	5.6	3.8	10.0	8.4
	13	27.3	16.8	6.7	4.3	12.5	10.2
	14	30.1	18.6	7.0	4.9	14.7	10.3
	Av.	24.4	15.4	5.9	4.1	11.4	8.8
T. occidentalis	16	17.5	10.9	4.7	2.9	11.7	8.8
	17	17.7	12.6	3.8	2.5	10.2	8.2
	18	19.6	12.2	5.0	3.1	12.2	10.3
	19	19.0	13.6	5.0	4.0	10.5	8.5
	20	17.4	11.4	4.0	2.7	11.2	9.1
	21	19.8	12.8	5.1	3.3	11.5	8.5
	22	19.2	10.6	4.5	2.5	10.9	7.0
	23	15.4	10.3	4.8	3.2	7.7	6.4
	24	27.3	20.8	4.4	3.3	15.6	12.5
	Av.	19.3	12.8	4.6	3.1	11.0	8.5
T. plicata	26	19.2	13.0	4.2	2.6	11.1	9.6
	27	17.4	12.7	3.8	2.8	11.6	10.8
	28	20.7	13.8	5.1	3.2	12.2	9.9
	29	14.3	10.1	3.4	2.4	10.8	9.5
	Av.	17.9	12.4	4.1	2.8	11.4	9.9

series with sister cambial cells of contrasting behavior, but also from other series in which both sister fusiform initials continued or both were lost. Hence the data represent the mean for all living and dying fusiform cambial cells in the various lineal series studied. In those cases where both sister cambial cells survived the extent of ray contact was determined at the midway point in the annual ring. In series where loss occurred measurements were made of both the enduring and failing initials at the last quarter point in the ring prior

to loss. The results are similar to those described above. In all 26 specimens studied the same general tendency was found. As a rule the persisting and enlarging fusiform initials had more extensive contacts with rays than the failing initials. This applied to all categories of comparison, namely the absolute number of ray cells, the number of rays, and the number of ray cells per mm. of length of the fusiform initials.

Fig. 9 illustrates the usual relationship. Here a pseudotransverse division toward the end of the growing season resulted in the production of sister cambial cells differing markedly in their ray connections. The lower sister, which had sparse ray contacts, was in turn transversely divided early in the following spring (Fig. 9*b*) and the lower portion soon disappeared. At the midway point through the annual ring the remaining part shrank (Fig. 9*c*) and shortly was lost from the cambium. Meanwhile the upper sister initial, which had much the best ray connections, underwent a sudden extension downward as the lower sister was lost and again (Fig. 9*d*) when another adjoining initial not shown in the figure vanished.

Some deviation from the general trend is of sporadic occurrence. Occasionally fusiform initials with poor ray associations continue and, conversely, some initials with extensive ray contacts are lost. One such uncommon instance is illustrated in Fig. 8. Fig. 8*a* shows the origin of sister cambial cells in an aestival pseudotransverse division. The upper sister soon lost contact with rays, this condition continuing without substantial improvement from spring (Fig. 8*b*) to summer (Fig. 8*c*) of the next year's growth. Despite the absence or sparsity of ray contacts the initial endured, although it is noteworthy that elongation was not as extensive as in the lower sister which had good ray connections. Most fusiform initials with sparse ray connections are soon lost from the cambium unless, as sometimes happens, their ray contacts are suddenly improved by loss of a neighboring fusiform initial.

The data in Table IV reveal some features of interest. On the average the combined height of the rays adjoining fusiform initials, expressed in number of ray cells, was similar in both species of *Chamaecyparis* and again in both species of *Thuja*. Also, the differences in ray contacts between enlarging fusiform initials on the one hand, and failing initials on the other, were comparable in the two genera. The order of difference was 57% in *Chamaecyparis* and 48% in *Thuja*. However, much fluctuation in the absolute measure of ray contact appears among the various samples. For instance, in the case of *Chamaecyparis thyoides* the extent of ray contact (number of ray cells) was low in tree No. 1 and high in tree No. 7. Likewise in *Thuja occidentalis* the ray connections of fusiform initials in tree No. 24 were nearly twice as extensive as those in tree No. 23. In both comparisons the differences are attributable to dissimilarities in the mean height rather than the frequency of the rays. The fluctuations in the average size of the ray contacts in various specimens, even though sometimes extensive, appear to have little or no bearing on general cambial behavior. But in the cambium of each specimen the behavior of individual fusiform initials as regards survival or loss is greatly

influenced by the *relative* sizes of their ray contacts. In other words, the fate of fusiform initials is determined more largely by the differences in extent of ray contacts among neighboring initials than by the prevailing size of the contacts.

Discussion

In the stems of trees ranging upward from a few inches in diameter the majority of the pseudotransverse divisions involved in the multiplication of fusiform cambial cells occur toward the end of the growing season. Many take place just prior to the production of the last late-wood elements, the partitioning anticlinal wall in many cases first appearing in the terminal cells of the annual ring. The fact that these cells, obviously formed after pseudotransverse division, have the general characteristics of late wood indicates that the transverse divisions must have occurred during the summer or early autumn. Only very rarely do pseudotransverse divisions happen during the development of the first-formed portions of early wood.

Aestival pseudotransverse division is often followed by considerable extension of the new-formed sister initials between consecutive xylemward periclinal divisions, particularly in the overlap between the sister initials. Although extensive this elongation is not necessarily rapid since the elapsed time is sometimes considerable and may include the winter resting period.

During the early growth of the following year elongation of the newly formed sister cambial cells is not extensive, total proximal and distal elongation usually being less during the development of the first quarter than in any of the succeeding quarters of the annual increment. Actual rate of elongation unfortunately is not known. Dendrographic studies on North American trees (Byram and Doolittle (4), Fraser (5), Friesner and Walden (6), Kienholz (7), Korstian (8), and MacDougal (9)), have revealed wide differences in the time of inception of cambial activity, the progress of radial increment, and the time of cessation of growth. Graphs in the literature show growth curves of varied patterns. For instance, according to the results of different investigators the time required for the production of the first quarter of the total annual increment (xylem, phloem, etc.) ranged from one-eleventh to one-fourth the elapsed period of growth during the year. Unfortunately the growth records of the trees studied in the present investigations are unknown. A few observations on local trees of *Thuja occidentalis* indicated that the first part of the annual ring was produced relatively quickly. In some cases approximately one-third of the xylem was already developed by mid-June. Although the elongation of fusiform initials during the production of the first quarter of the annual ring was not extensive, in at least some specimens the actual rate of elongation possibly exceeded that later in the growing season.

During the deposition of the second, third, and final quarters of the annual ring, fusiform initials tended to elongate by increasing amounts. It is probable that in most specimens the elapsed time required for the development of the successive quarters of the annual ring was also progressively longer.

The multiplication of fusiform cambial cells is accompanied by loss, the latter sometimes occurring to such extent that the net gain in number of fusiform initials is slight. In general, the loss of fusiform initials tends to increase toward the latter part of the growing season. At this time the surviving fusiform initials experience their greatest elongation, spurts in elongation being coincident with loss of neighboring initials.

The pattern of cells in the cambium is not static. Rather it undergoes much change, particularly during the development of the last quarter of the annual ring. While certain initials elongate extensively others "shrink" and lapse into maturation or are subdivided to smaller meristematic units some destined to become ray initials. The differences in behavior as exemplified by the enlargement of certain initials and the simultaneous contraction of their neighbors apparently stem from differing physiological conditions within the cells in question, the internal circumstances being influenced by the local environment. For instance, it would appear that the failing cells were unable to maintain the usual turgidity in competition with their enlarging neighbors.

One of the most obvious of the variables in the local environment of each fusiform initial is the extent of ray contact. This varies greatly from one fusiform initial to another. Data reveal the existence of a general tendency for the fusiform initials which are largest and have the most extensive ray associations to survive and elongate, while adjoining initials with poor ray contacts undergo size reduction and maturate into abnormal permanent cells or are subdivided to potential ray initials. Demands on water, food materials, and such other substances as are required for growth must be very great in the cambial zone during the growing season. Possibly most of these materials reach the initiating portion of the cambium mainly through the cambial links of the vascular rays. If such be the case those particular fusiform cambial cells with the most extensive ray associations are in a favored position in competition with their neighbors.

The elongation of fusiform initials results in progressive change in their ray associations. As a consequence of the increasing overlap between elongating initials the extent of ray contact tends to decrease relative to the length of the fusiform initials. Further alterations in the ray contacts follow pseudo-transverse division. Rarely do the resulting sister initials have exactly similar amounts of ray association. Often one of the fusiform initials has notably deficient ray contacts as compared with its sister initial. As a general rule the fusiform initials with poor ray contacts lag in competition with their neighbors and soon maturate to imperfect permanent elements or more often become subdivided to smaller units. The reduction of the initials with sparse ray contacts, usually to smaller meristematic units some of which survive to initiate new rays, aids in the eventual rectification of the ray shortages in those particular areas.

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ANTAGONISME DES MICROORGANISMES DU SOL ENVERS LE *COLLETOTRICHUM LINICOLA* AGENT DE L'ANTHRACNOSE DU LIN[1]

PAR RENÉ-O. LACHANCE[2]

Résumé

L'auteur a fait un inventaire de la flore microbienne de certains sols du Bas Saint-Laurent où se cultive le lin. Un certain nombre des microorganismes isolés se sont avérés antagonistes *in vitro* à *C. linicola*.

Plus de la moitié eurent une action fongicide en milieu solide, tandis que les autres n'eurent qu'un effet fongistatique après quatre jours de croissance continue.

En milieu solide, quelques bactéries ont provoqué une germination anormale des spores et une croissance anormale du mycélium. En milieu liquide, une seul de ces bactéries a permis la germination des spores; les autres ont eu une action antagoniste plus prononcée en milieu liquide qu'en milieu solide. Les structures anormales sont décrites et illustrées.

L'influence du milieu de culture sur l'action antagoniste des microorganismes fut étudiée. De douze milieux mis à l'essai, les géloses aux patates, à l'oignon, et la gélose nutritive sans dextrose se sont avérées les plus utiles.

Les études *in vivo* ont indiqué qu'aucun des antagonistes utilisés n'a controlé l'anthracnose du lin, qu'il s'agisse de graines saines, semées en terrain contaminé ou de graines contaminées, semées en terrain sain, que l'introduction des antagonistes ait été faite directement dans le sol ou par la bactérisation de la graine. Toutefois, lorsque la terre fut préalablement stérilisée à l'autoclave, la moitié des organismes ont diminué le pourcentage d'anthracnose, d'autres n'ont apporté aucun changement, tandis que d'autres moins nombreux l'ont augmenté.

Introduction

L'influence de la flore microbienne du sol sur l'activité de plusieurs pathogènes des plantes fut l'objet d'études nombreuses au cours des dernières décennies.

En plus de l'intérêt académique qu'offrent ces études, elles ont une portée pratique considérable, car pour nombre de maladies des racines il n'existe d'autre remède efficace que la lutte biologique, laquelle consiste à favoriser le développement d'organismes antagonistes à ces pathogènes. Cette méthode de lutte se pratique avec succès, du reste. Il paraît bien établi que c'est grâce à l'activité accrue des microorganismes du sol que l'engrais de ferme ou l'engrais vert empêchent le développement à l'état épidémique de certaines maladies des racines (2, 4). Certains chercheurs ont essayé d'incorporer au sol, soit par la bactérisation des semences, soit par l'ensemencement direct, des organismes dont la valeur comme antagonistes spécifiques avait été éprouvée *in vitro* (12, 7, 8).

La revue des travaux publiés sur ce sujet a été faite par les auteurs suivants: Garret (3), Waksman (13), D'Aeth (1) et Weindling (14).

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Contribution N° 1085 de la Division de Botanique et de Phytopathologie, Service Scientifique, Ministère de l'Agriculture, Ottawa, Canada.

[2] *Phytopathologiste, Laboratoire Fédéral de Phytopathologie, Ste-Anne-de-la-Pocatière, Qué.*

Durant la dernière guerre, la culture du lin a joui d'une popularité inaccoutumée dans la province de Québec. Les maladies du lin ont subitement pris une importance particulière, et quelques-unes, comme l'anthracnose causée par *Colletotrichum linicola*, ont menacé sérieusement cette culture. Comme corollaire aux autres phases de portée pratique plus immédiate (5, 6, 9), l'auteur a fait une étude sur l'antagonisme, envers ce parasite, de microorganismes isolés de divers sols où se cultive le lin dans le Bas Saint-Laurent. Quelque négatifs que soient les résultats de ces recherches du point de vue de la lutte contre l'anthracnose, il paraît utile de les présenter dans l'espoir qu'ils permettent à d'autres chercheurs d'éviter des labeurs inutiles.

Travail expérimental

Les travaux poursuivis comprennent trois phases bien distinctes: la première est l'inventaire de la flore microbienne des sols quant à la présence d'organismes antagonistes à *C. linicola*, la deuxième, l'étude *in vitro* de l'action des antagonistes sur le pathogène, et, finalement, l'essai *in vivo*, en serre, des meilleurs antagonistes en vue de la lutte contre l'anthracnose.

Inventaire

Des échantillons de sol furent prélevés aussi aseptiquement que possible à trois périodes différentes au cours de la saison de végétation de 1948, soit, lorsque les plants avaient de 4 à 5 pouces de hauteur au début de juin, puis, vers le 15 juillet, au début de la floraison, et, finalement, au temps de la récolte, au début de septembre.

L'analyse bactériologique fut faite le plus tôt possible après l'échantillonnage. Le plus grand nombre possible d'espèces de bactéries, d'actinomycètes et de champignons furent isolés. A cette fin, divers milieux furent utilisés: la gélose aux pommes de terre à pH 6.2 et pH 4.0, le milieu Thornton à l'asparagine et au mannitol à pH 7.0, et l'agar nutritif à pH 6.4. Les espèces de bactéries et de champignons isolés furent éprouvés en boîtes de Petri pour leur valeur antagoniste envers *C. linicola*. Après plusieurs essais préliminaires, la technique suivante fut adoptée. Les microorganismes furent ensemencés sur gélose aux pommes de terre en boîtes de Petri placées dans l'incubateur à 25° C. durant 24 heures, puis vaporisés avec une suspension de spores de *C. linicola* dans de l'eau stérile. Les boîtes furent ensuite laissées à la température du laboratoire. La valeur des différents microbes isolés du sol comme antagonistes fut évaluée par la largeur de la zone d'inhibition. Du nombre imposant de microorganismes isolés du sol, environ 26 se sont montrés antagonistes *in vitro*. Les bactéries et les actinomycètes étaient en nombre à peu près égal, tandis que seulement un très petit nombre de champignons manifestèrent quelque antagonisme.

Étude in vitro

Le comportement des spores de l'agent pathogène dans la zone d'inhibition fut observé au microscope. L'effet des antagonistes s'est manifesté par une inhibition totale de la germination des spores ou par un arrêt après un début

plus ou moins prononcé. Ces spores non germées ou dans un état statique étaient mortes dans la moitié des cas au moins, comme le prouvera une expérience rapportée plus loin. En dehors de la zone d'inhibition, la germination des spores et la croissance du mycélium parut se faire normalement, sauf avec cinq organismes qui ont manifesté leur antagonisme en ne permettant qu'une croissance limitée et très anormale de l'agent pathogène.

Les bactéries 363 et 421 ont permis une croissance limitée des tubes germinatifs dans la zone d'inhibition et ont provoqué la formation de renflements de ces tubes à intervalles irréguliers (Fig. 1). Ces renflements étaient présents,

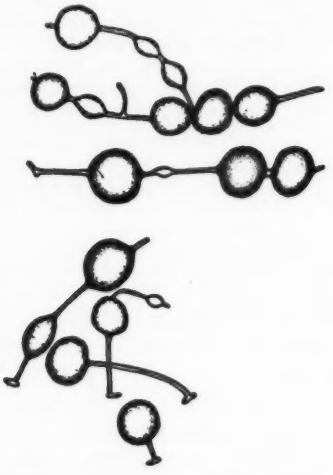

FIG. 1. Spores de *C. linicola* en germination dans la zone d'inhibition produite par les bactéries 363 et 421.

même en dehors de la zone d'inhibition où la croissance du mycélium de *C. linicola* était abondante. Des malformations analogues ont été décrites par Porter (10) pour une espèce d'*Helminthosporium*.

Les bactéries 365, 420 et 423 ont également incité une germination anormale. Au lieu de produire un tube germinatif, les spores donnèrent naissance à des cellules très courtes, irrégulières, et à parois épaisses (Fig. 2). Chaque colonie issue d'une spore se développa, mais ne s'étendit pas suffisamment pour rejoindre les limites de la colonie adjacente.

Viabilité des spores de C. linicola situées dans la zone d'inhibition

Afin de déterminer si les bactéries produisent une substance fongistatique ou fongicide, de petits fragments de gélose furent prélevés dans la zone d'inhibition et repiqués sur tubes de gélose, 6, 24, 48 et 96 heures après l'ensemencement de *C. linicola*. A l'étude des résultats consignés dans le Tableau I, on constate que plus de la moitié des organismes étudiés secrètent une substance

fongicide, tandis que les autres n'ont qu'un effet fongistatique. D'autre part, cette action ne s'exerce qu'après une période de deux ou même de quatre jours dans certains cas. Certains organismes ne figurent pas au tableau, parce qu'ils ne produisirent pas une zone suffisamment large.

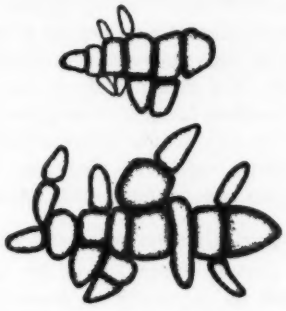

FIG. 2. Germination anormale des spores de *C. linicola* en présence des bactéries 365, 420 et 423.

TABLEAU I

EFFET DES ANTAGONISTES SUR LA VIABILITÉ DES SPORES DE *C. linicola* DANS LA ZONE D'INHIBITION

N° de la culture antagoniste	Viabilité des spores après:			
	6 heures	24 heures	48 heures	96 heures
348	+	+	−	−
350	+	+	+	−
352	+	+	+	−
353	+	−	−	−
355	+	+	+	+
358	+	+	+	+
359	+	+	−	−
360	+	+	−	−
363	+	+	+	−
365	+	+	+	+
366	+	+	+	+
418	+	+	+	+
421	+	+	+	+
422	+	+	−	−
423	+	+	+	+

Action des antagonistes en culture liquide

Afin de suivre de plus près le comportement des spores de *C. linicola* en présence des antagonistes, des études furent faites en cellules Van Teighem. Les antagonistes furent cultivés dans une décoction de pommes de terre contenant un pour cent de dextrose et un pour cent de levulose. Une anse d'une suspension de spores de *C. linicola* fut ajoutée à une goutte de culture

microbienne montée en cellule Van Teighem. Les observations furent faites à intervalles rapprochés durant la première journée et une fois par jour durant les cinq jours suivants. Des études analogues furent faites avec le filtrat des mêmes cultures avant et après un chauffage de dix minutes à l'eau bouillante. Pour la filtration, l'appareil Seitz fut utilisé.

En général, aucune germination n'a eu lieu en présence des bactéries et les parois des spores étaient le plus souvent désagrégées, tandis que le protoplasme paraissait coagulé. En présence de la bactérie 363, les spores de *C. linicola* ont germé de la même façon que sur gélose (Fig. 1), tandis qu'avec les bactéries 365, 420, 421 et 423, aucune germination n'eut lieu, alors que sur gélose une germination anormale avait été observée. En présence des Actinomycètes et des champignons la germination fut normale, bien que fort lente.

Influence du milieu sur la production des substances antibiotiques

Certaines bactéries à l'étude eurent, dans les essais préliminaires, une croissance tellement rapide qu'ils couvrirent toute la surface de la boîte de Petri en moins de vingt-quatre heures, de sorte qu'il devenait difficile, sinon impossible, d'apprécier leur valeur comme antagonistes. Afin de trouver quels seraient les meilleurs milieux de culture pour déterminer le pouvoir antibiotique des microorganismes isolés des sols, douze milieux furent mis à l'essai, dont huit d'origine organique végétale, deux d'origine organique animale et deux de nature synthétique. Tous les organismes mis à l'essai furent cultivés en triplicata sur chacun des milieux. Le Tableau II donne les résultats de cet essai.

De ces données, on peut conclure que pour juger du pouvoir antagoniste d'un organisme, on doit le mettre à l'essai sur plusieurs milieux. Les trois qui se sont avérés les plus convenables dans la majorité des cas sont la gélose aux pommes de terre à un pH de 6.2, la gélose nutritive Difco (extrait de boeuf et peptone) sans dextrose, et la gélose aux oignons.

Certains organismes ont produit une zone imposante sur un seul milieu, alors que d'autres furent beaucoup moins sélectifs. De plus, les organismes qui avaient envahi toute la surface de la boîte de Petri sur gélose aux pommes de terre ont fait une croissance normale sur d'autres milieux, ce qui facilita l'évaluation de leur antagonisme.

Essais in vivo des meilleurs antagonistes

Ces essais furent poursuivis en serre dans de la terre argileuse, parce que c'est de ce type de sols que la majorité des bactéries mises à l'essai avaient été isolées. La variété Stormont Cirrus fut employée pour ces essais.

Pour contaminer le sol avec *C. linicola*, la méthode préconisée par Riker et Riker fut adoptée en substituant du blé au mélange de sable et de farine de maïs.

Comme il fut impossible de se procurer de la graine de lin naturellement infectée, de la graine enregistrée fut vaporisée avec une suspension de spores de *C. linicola* et séchée à l'air avant d'être utilisée.

TABLEAU II

EFFET DE LA COMPOSITION DU MILIEU SUR LA DIMENSION DE LA ZONE D'INHIBITION

Antagonistes	Czapek	Elliott	Oignon	Gruau d'avoine	Gélose nut. + dextrose	Leonian	Haricot	Pois	Gélose nutritive	Maïs	Gélose patates	Malt
348	9	9	—	0	2	0	3	0	5	0–5	—	—
350	0	0	2	6	0	Tr.	2	3	6	—	3	Tr.
352	6	0	4	6	6	7	6	5	12	0	Env.	3
353	5	7	24	0	10	0	3	0	6	10	10–17	6–10
355	0–4	0–4	0–2	0	9	0	3	2–7	8	0	3	0
358	—	—	0	2	Tr.	—	Env.	1	1	0	0	—
359	Tr.	Tr.	4	3–8	6	5	4	3–6	3	2	3	—
360	3	—	5	1	4	3	5	4	8	0–4	3	3
362	5	2	4	6	6	8	5	4	9	5	4	3
363	0	0	Tr.	5	Tr.	0	4	3	4	0	0	—
364	0	0	0	0	3	0	1	2	15	0	Tr.	—
365	0	0	0	2	0	Tr.	1	0	4	0	0	0
366	4	2	3	3	2	2–5	2	3	4	0	Env.	0
418	0	0	0	0	3	0	0	2	0	—	Tr.	—
419	0	6	4	5	0	4	4	6	10–15	2	5	—
420	2	2	0	4	0	Tr.	4	0	4	0	0	0
421	0	0	Tr.	3	3	2–4	0	1	2	0	2	—
422	10	10	4	6	8	9	6	6	10	7	6	6
423	1	1	0	4	0	0	0	0	3	1	0	0
Témoin	1	1	4	3	2	3	2	2	1	4	4	4

0 à n = *Indique la largeur en mm. de la zone d'inhibition.*
— = *L'antagoniste a refusé de croître sur ce milieu.*
Env. = *L'antagoniste a couvert toute la surface de la boîte, en moins de vingt-quatre heures.*
Tr. = *Zone apparente mais trop étroite pour être mesurée.*

L'introduction des bactéries antagonistes fut faite suivant deux méthodes: la première, par inoculation directe du sol au moyen d'un vaporisateur De Vilbiss avec une culture liquide des bactéries à l'étude, et la seconde, par bactérisation de la semence. Le procédé de bactérisation de la semence fut en tout point identique à celui qu'on utilise pour inoculer les graines de légumineuses. De la tourbe finement moulue ayant un pH de 7.0 fut inoculée avec une culture liquide de bactéries à raison de 40 cc. par 100 gm. de tourbe. Dans les premières expériences, il a fallu, faute d'espace, limiter le nombre des antagonistes à quatre, mais un cinquième traitement fut ajouté qui consistait en un mélange de douze bactéries toutes antagonistes *in vitro* à *C. linicola*. Pour opérer le mélange, les bactéries furent cultivées séparément en milieu liquide, et des quantités égales de chacune d'elles furent utilisées pour humecter la tourbe.

La viabilité des organismes dans cette tourbe fut vérifiée par des cultures en boîtes de Petri avant de procéder à la bactérisation. La graine de lin fut ensuite humectée, enrobée avec la tourbe inoculée et semée immédiatement en serre.

Des pots de six pouces de diamètre furent utilisés, et environ deux cents graines furent semées dans chaque pot. Les divers traitements furent faits

en quadruplicata. Les plantes furent arrachées lorsqu'elles avaient de deux à trois pouces de hauteur, et le pourcentage de plants atteints d'anthracnose fut alors déterminé.

Dans les Tableaux III, IV, V, et VI, les pourcentages inscrits ont été transformés en degrés d'après la table de Bliss (11), pour fins d'analyse statistique. Seules les bactéries furent mises à l'essai au cours des divers tests *in vivo*. Les autres microorganismes ont été éliminés, faute d'espace en serre.

TABLEAU III

EFFET DE LA BACTÉRISATION DE SEMENCE SAINE SEMÉE EN TERRAIN CONTAMINÉ. MOYENNE DE QUATRE REPLICATA

Traitements	% d'anthracnose en degré angulaire	
Témoin	43.8	10.22
Bactéries 348	61.7	
353	41.2	
419	43.3	
422	35.7	
Mélange de 12 bactéries	32.5	
D.R.S. *P*, 0.05	15.4	

TABLEAU IV

EFFET DE LA BACTÉRISATION DE LA SEMENCE DE LIN INFECTÉE DE *C. linicola* SEMÉE EN TERRAIN SAIN

Traitements	% d'anthracnose en degré angulaire	
348	22.79	5.87
Témoin	29.45	
Mélange	35.18	
419	36.22	
353	40.16	
422	43.65	
D.R.S. *P*, 0.05	8.65	
P, 0.01	12.24	

Effet de l'antagonisme dans le cas d'une semence saine bactérisée, semée en terrain contaminé

Afin de déterminer si la bactérisation d'une semence saine peut protéger celle-ci contre les attaques du *C. linicola* présent dans le sol, de la terre argileuse fut inoculée suivant la méthode décrite précédemment, et maintenue bien humide en serre durant une semaine, afin de permettre au *C. linicola* de s'établir dans le sol. De la graine saine fut ensuite bactérisée avec les quatre

meilleurs antagonistes et le mélange de douze autres bactéries, et semée en pots de six pouces. Les plants furent arrachés lorsqu'ils eurent atteint deux ou trois pouces de hauteur. Le Tableau III donne le pourcentage de plants atteints d'anthracnose.

TABLEAU V

ACTION DES ANTAGONISTES INTRODUITS DIRECTEMENT DANS LE SOL

Traitements	% d'anthracnose, moyenne de quatre répétitions			
	Semis immédiat		Semis différé	
348	28.84	4.67	14.33	3.72
353	27.12		13.18*	
419	14.07		13.41*	
422	34.89		20.71	
Mélange	31.60		18.50	
Témoin	31.16		19.80	
D.R.S. *P*, 0.05	7.12		5.61	

* *Significatif.*

TABLEAU VI

ACTION DES ANTAGONISTES SUR UNE SEMENCE CONTAMINÉE, SEMÉE EN TERRE STÉRILISÉE

Traitements	% d'anthracnose, moyenne de quatre répétitions	
348	27.93	6.91**
419	34.68	**
418	42.38	**
422	42.50	**
362	43.39	**
353	47.29	*
366	50.38	
Témoin	59.16	
359	60.48	
349	68.10	
358	70.08	
352	75.25	**
355	82.75	**
D.R.S. *P*, 0.05	11.54	
P, 0.01	15.48	

* *Significatif.*
** *Très significatif.*

Aucune des bactéries utilisées n'a diminué d'une façon significative le pourcentage des plants malades. La bactérie 348 a même augmenté sensiblement le nombre de plants malades.

Effet de l'antagonisme dans le cas d'une semence contaminée et bactérisée, semée en terrain sain

La graine de lin fut artificiellement infectée puis bactérisée avec de la poudre de tourbe inoculée avec les mêmes organismes que précédemment, soit 348, 353, 419, 422 et le mélange des douze bactéries. Le nombre de plants malades fut compté. Le Tableau IV donne les résultats obtenus. Aucune des bactéries utilisées n'a diminué de façon significative le pourcentage de plants atteints d'anthracnose.

Effet de l'antagonisme dans le cas de semence contaminée, semée en terrain inoculé avec les antagonistes

Dans le but de déterminer si l'inoculation directe du sol est plus ou moins efficace que la bactérisation de la semence pour combattre l'anthracnose, la couche supérieure de trois pouces d'épaisseur fut enlevée des pots, finement pulvérisée, et finalement arrosée au moyen d'un vaporisateur avec une culture liquide des bactéries à l'étude; après l'opération le sol était bien humecté. Les mêmes organismes 348, 353, 419, 422 et un mélange de douze autres bactéries furent soumis à cette expérience. Pour chacun des traitements, il y eut huit répétitions; toutefois, quatre répétitions furent ensemencées immédiatement, alors que pour les quatre autres, l'ensemencement fut différé de quinze jours pour permettre aux bactéries de s'établir dans le sol et déterminer si l'action des bactéries peut être plus efficace lorsqu'on leur permet de se multiplier. Les plants furent arrachés lorsqu'ils avaient deux ou trois pouces de hauteur, et le pourcentage de plants malades fut alors déterminé. Les résultats apparaissent au Tableau V où l'on voit qu'aucun traitement ne fut efficace lorsque la graine fut semée immédiatement après l'inoculation du sol. Par contre, lorsque le semis fut différé de quinze jours, deux bactéries 353 et 419, ont réduit de façon significative le pourcentage d'anthracnose.

Effet de la bactérisation de semence contaminée, semée en terre stérilisée

Devant les résultats des expériences précédentes, il parut intéressant de chercher une explication au comportement différent des organismes *in vitro* et *in vivo*. A cette fin, la terre fut stérilisée à l'autoclave à quinze livres de pression pendant une heure. La graine de lin fut inoculée et bactérisée suivant la méthode déjà décrite. Toutefois, le nombre d'organismes mis à l'essai fut de douze au lieu de quatre; les huit bactéries ajoutées furent choisies parmi les douze qui constituaient le mélange dans les expériences précédentes. Les plants furent arrachés lorsqu'ils avaient deux ou trois pouces de hauteur et le pourcentage de plants atteints d'anthracnose déterminé. Les résultats obtenus sont groupés dans le Tableau VI.

Ce tableau montre que six des bactéries à l'essai ont diminué sensiblement le pourcentage d'anthracnose, tandis que quatre autres n'ont apporté aucun changement important et que deux l'ont augmenté.

Discussion et conclusions

Du nombre considérable de microorganismes qui constituent la flore des sols où se cultive le lin dans le Bas Saint-Laurent, P.Q., quelques-uns se sont avérés antagonistes à *C. linicola*, agent de l'anthracnose du lin. L'effet des antagonistes est dû à la production d'une substance fongicide dans certains cas et fongistatique dans d'autres. L'action des bactéries antagonistes est plus marquée en milieu liquide qu'en milieu solide, à cause, vraisemblablement, d'une diffusion plus rapide et plus facile de la substance antibiotique.

Devant les différences que manifestent les diverses espèces isolées selon le milieu sur lequel on les cultive, il est permis de croire que bon nombre de celles qui sont rejetées aux épreuves éliminatoires ne le seraient pas s'il était possible de les mettre à l'essai sur un plus grand nombre de milieux artificiels.

Le comportement des spores de l'agent pathogène en gouttes pendantes et en présence des bactéries antagonistes confirme le fait que plusieurs bactéries produisent une ou des substances fongicides. La désagrégation des parois cellulaires et la coagulation du protoplasme sont, en effet, très marquées sous l'influence d'un bon nombre de bactéries. Il reste cependant que les substances toxiques produites par les bactéries étudiées ne sont pas stables à moins d'avoir été plus ou moins absorbées par le filtre utilisé.

En considérant les résultats obtenus en terre non stérilisée, on constate leur peu de concordance avec ceux obtenus *in vitro*. Deux explications se présentent naturellement à l'esprit; d'abord, l'antagonisme de la flore normale du sol utilisé vis-à-vis des organismes introduits dans le sol, puis, en second lieu, la possibilité que les nouveaux venus n'aient pas trouvé dans le sol où ils ont été introduits des conditions leur permettant de vivre et de se multiplier, car on sait que le sol est un milieu excessivement complexe et variable. Or, pour avoir été isolés de divers sols, ces microorganismes n'ont pas nécessairement trouvé dans le sol utilisé les conditions favorables à leur multiplication, ou, encore, à la production de substance antibiotique. Car tout n'est pas de croître: une expérience *in vitro* nous a démontré l'importance du milieu sur la production de substances antibiotiques. Il est probable toutefois que l'antagonisme des microorganismes du sol lui-même envers les bactéries inoculées soit le facteur qui ait empêché l'action antagoniste de ces derniers en prévenant leur croissance et leur multiplication.

L'expérience poursuivie en terre stérilisée nous démontre en effet qu'une fois la compétition disparue, la moitié des douze microorganismes introduits dans le sol y ont exercé une action antagoniste envers *C. linicola*. Il est probable que la flore normale de ces sols, éliminée par la stérilisation, exerçait plus qu'un effet de compétition, un véritable effet antagoniste. Quant aux quatre bactéries qui n'ont causé ni diminution, ni augmentation du pourcentage d'anthracnose, l'explication la plus plausible semble être qu'ils n'ont pu s'établir dans le sol. L'action synergétique des deux derniers, toutefois, est plus difficile à expliquer. Il arrive que les substances antibiotiques

produites par des antagonistes exercent à dose très faible une action stimulatrice, et il n'est pas impossible que, dans le sol utilisé et dans les conditions de l'expérience, ces deux bactéries aient produit très peu de substance antibiotique et que celle-ci aît agi comme stimulant et aît favorisé *C. linicola*. Il est également possible que, dans le sol, ces bactéries aient produit une substance tout-à-fait différente de celle qu'elles produisent *in vitro* et que cette substance ait eu un effet stimulant.

Si l'on envisage le point de vue pratique, la bactérisation des semences comme moyen de lutte biologique contre l'anthracnose est inférieure à la lutte chimique actuellement en honneur. Toutefois, la lutte biologique a une importance beaucoup plus grande lorsque l'on considère les maladies dues à des organismes dont l'habitat est le sol. Pour beaucoup d'entre elles, il n'existe pas de moyen de lutte efficace et pratique à part la création de variétés résistantes.

English Summary

Several soils where flax is grown in the Lower St. Lawrence district were surveyed for the antagonistic value of their microflora towards *Colletotrichum linicola*. A certain number of the organisms isolated from these soils were antagonistic *in vitro* to *C. linicola*.

More than half of these organisms acted as fungicides while the others exerted a fungistatic action after being in contact with the spores during four days.

On solid media a few antagonistic bacteria have induced abnormal spore germination and mycelial growth while in liquid culture the same type of germination was observed with only one of these bacteria. The abnormal structures are described and illustrated.

The influence of culture media on the production of antibiotic has been studied. Out of 12 media used, potato dextrose agar, nutrient agar, and onion agar were found to be the most useful.

In vivo studies under greenhouse conditions have shown that none of these antagonists has controlled flax anthracnose unless the soil was previously sterilized. In this latter case, half of the organisms tested have significantly reduced the percentage of anthracnose. Some have brought no change while a few have increased the percentage of diseased plants.

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Contents

Page

Studies on Fungus Parasites of Strawberry Leaves in Ontario—*Joan Fall* - 299

Activity of Antibiotics Against *Ascochyta pisi*—*V. R. Wallen and A. J. Skolko* 316

Two New Smuts on *Carex* in Canada—*D. B. O. Savile* - - - - - - - 324

Uptake of P^{32} by Some Cereals—*J. M. Naylor, G. W. R. Walker, D. Murray, C. O. Person, J. W. T. Spinks, and T. J. Arnason* - - - - - - - - - 329

Effect of Pasmo Disease of Flax on the Yield and Quality of Linseed Oil—*W. E. Sackston and R. B. Carson* - - - - - - - - - - - - 339

Studies on the Production of Vitamin B_{12}-active Substances by Micro-organisms—*Margaret O. Burton and A. G. Lochhead* - - - - - - - 352

Studies on the Legume Root Nodule Bacteria. I. Detection of Effective and Ineffective Strains—*D. C. Jordan and E. H. Garrard* - - - - - - - 360

Observations on Quaternary Ammonium Disinfectants—*C. E. Chaplin* - - 373

Physiological and Biochemical Studies in Plant Metabolism. III. The Effects of Starvation and Mechanical Stimulation on the Respiratory Metabolism of the First Leaf of the Wheat Plant—*D. W. A. Roberts* - 383

Biochemistry of the Ustilaginales. V. Factors Affecting the Formation of Ustilagic Acid by *Ustilago zeae*—*J. A. Thorn and R. H. Haskins* - - - 403

Studies of Virus Diseases of Strawberries in British Columbia. II. The Separation of the Component Viruses of Yellows—*Frances C. Mellor and Randal E. Fitzpatrick* - - - - - - - - - - - - - - - - 411

The Annual Cycle of Size Changes in the Fusiform Cambial Cells of *Chamaecyparis* and *Thuja*—*M. W. Bannan* - - - - - - - - - - - - 421

Antagonisme des Microorganismes du Sol Envers le *Colletotrichum linicola* Agent de l'Anthracnose du Lin—*René-O. Lachance* - - - - - - - 438

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